CHAPTER VI - DISCUSSION

CHAPTER VI

DISCUSSION

VI-1 CLONAL PROPAGATION OF ELITE TREES

VI-2 EFFECT OF CULTURAL PARAMETERS ON GROWTH OF *COMMIPHORA WIGHTII* CELL CULTURES

VI-3 EFFECT OF CULTURAL PARAMETERS ON PRODUCTION OF β-C-3 STEROLS AND GUGGULSTERONES

VI.

DISCUSSION

The results of the experiments conducted to realise the objectives stated as in Introduction (Chapter I-3) are described in the earlier chapters (Chapters III, IV and V). To what extent the evidences obtained in the present investigations have realised the aims and objectives now remains to be assessed.

The results obtained in this presuit are discussed in the present chapter, with reference to other reports under the following captions :

VI-1 CLONAL PROPAGATION OF ELITE TREES

VI-2 EFFECT OF CULTURAL PARAMETERS ON GROWTH OF COMMIPHORA WIGHTII CELL CULTURES.

VI-3 EFFECT OF CULTURAL PARAMETERS ON PRODUCTION OF β-C-3 STEROLS AND GUGGULSTERONES.

VI-1. CLONAL PROPAGATION OF ELITE TREES

To our knowledge, this is the first report of *in vitro* techniques applied to the genus *Commiphora*. Shoot multiplication in *Commiphora wightii* apparently occurs as a result of the release of axillary buds or meristems from apical dominance or dormancy, as it is common in many woody species (Lane, 1979; Gupta *et al.*, 1980; Lineberger, 1983; Sutter and Barker, 1985).

The present study provides a method that ensures a multiple shoot induction from juvenile and adult tree shoot tips of *Commiphora wightii* and the subsequent rooting of shoots regenerated in culture. Although, differences existed between the behaviour of seedling and mature material in culture, the use of seedling material for determining the proper conditions for culture of mature material has certain advantages and is recommended when one is first trying to propagate a difficult woody species *in vitro*. Ease of obtaining sterile material, tolerance to a wider range of nutrient media and growth conditions are advantages of using seedling material. The mature elite material, which is usually less plentiful, can be saved for critical determination of media and other culture conditions. In the present investigations, the method used for the propagation of juvenile material was applicable to the mature trees with some modifications.

The *in vitro* behaviour of the juvenile as well as mature material is discussed in the following pages in relation to the available literature relevant to the different experiments conducted with this plant.

Identification of Elite Trees :

The *in vitro* propagation of many herbaceous plants has been successfully accomplished (Conger, 1981). Considerable amount of work has been done in fruit tree propagation (Skirvin, 1981). Similar advances have not been reported for the propagation of economically important forest trees (Mott, 1981). A method for the rapid vegetative propagation of elite genotypes of forest trees would be of great value in afforestation programme. (Fielding, 1983).

Datta *et al.* (1983) clonally multiplied the mature genotypes of *Dalbergia sissoo*. Tree selection and establishment of progeny trials has been accomplished with the genus *Eucalyptus* (Gupta *et al.*, 1981; Rao and Venkateswara, 1985; Gupta *et al.*, 1983). Gupta *et al.* (1980) reported the clonal multiplication of *Tectona grandis* trees which are 50-100 years old and have wood of good quality and were identified as elite trees among the teak forests in several regions of India. Some other reports on selected genotypes in tree species are on *Liquidambar styraciflua* (Sutter and Barker, 1985), rare species *Stylidium*

coroniforme and other Stylidium species (McComb, 1985), Leucaena leucocephala (Dhawan and Bhojwani, 1985).

In Commiphora wightii, no systematic screening of trees growing wild in the forests in the different regions of arid and semi-arid zones of Gujarat has been done. We have standardised procedures for the extraction of desired steroids both from plant parts and exuded gums. Using UV-spectrophotometric methods (Chapter II-6.a.1), qualitative and quantitative estimates were made. Elite trees were chosen on the basis of the percentage of the ketonic fraction and fraction containing the two sterones (Guggulsterone -Z and -E).

Fortyeight trees from the three agroclimatic regions/areas (Vasad, Sindhrcat and Kutch) were screened. Our results (Chapter III-1) clearly indicated genotypic variation with respect to the % ketonic and % guggulsterone fraction in the trees. No correlation was however, observed in the level of ketonic and guggulsterone fraction in the gum exudate and the different agroclimatic conditions under which the trees were grown.

Results presented in the Chapter III-2 reveal that the highest percentage of sprouting as well as survival of the cuttings was obtained with 'Seradix 3' treatment. However, only thick branches (1 cm diameter) survived after sprouting

indicating plentiful amount of material from elite trees for the *in vivo* propagation. Such severe pruning is harmful for the selected genotypes. Similarly, while trying to raise plantlets from the seeds, the percentage of germination was found to be very low (8 to 10%). Furthermore, the seed raised population would be highly heterozygous and therefore, it has no value for clonal propagation of proven genotypes.

Considering the above mentioned problems, attempts were made to clonaly propagate the trees by employing shoot tip culture technique.

Since the seeds of *Commiphora wightii* were collected from forest, they required a complex sterilization regime (Chapter II - 3.a.1). Treatment with 10 ppm solution of Nystatin, the systemic fungicide with streptomycin and double sterilization of the seeds was found absolutely essential to get sterile cultures.

Dhawan and Bhojwani (1985) sterilized the seeds of *Leucaena leucocephala* twice with 1% sodium hypochlorite solution before and after imbibition. McComb (1985) applied a novel method for sterilization of seeds of *Stylidium coroniforme* by placing the seeds in a syringe with a millipore filter attached and by shaking in 5% sodium hypochlorite.

It is evident from the observations that BAP (optimum at 4 mg/l) is the suitable cytokinin for proliferation of shoots (Chapter III-3.b). The superiority of BAP over other cytokinins for multiple shoot formation in woody species has also been observed by other investigators (Monette, 1986; Brand and Lineberger, 1986; Paily and D'Souza, 1986; Goyal *et al.*, 1985; Chalupa, 1984). For *Gardenia* (Economou and Spanoudaki, 1985) 2-ip was found to be the most effective for shoot proliferation.

Highest frequency of shoot formation (80%) with multiple shoots was registered when Kn and BAP were used together at 4 mg/l, than either of the cytokinins used separately in the medium. The highest frequency of bud breaking in BAP-Kn medium might be due to the synergism between the cytokinins (Chapter III-3.b.).

Combination of BAP and Kn also showed best results for multiple shoot formation in other woody species of fruit, for example, *Annona squamosa* (Nair *et al.*, 1984), *Punica granatum* Linn. (Iyer, 1984); *Tamarandus indica* (Gupta *et al.*, 1981), and ornamental (Williams *et al.*, 1984) plants. Gupta *et al.* (1980, 1981) reported low levels of Kn and BAP for multiple shoot formation in teak and *E. citriodora*.

In *Commiphora wightii*, auxins alone induced only callus formation; while along with cytokinins, callus was

accompanied by shoot formation. Such auxin response sometimes with inhibitory effect on shoot formation, has been reported in many cases. Callusing at the cut end of the buds was observed in Eucalyptus citriodora and Tectona grandis, when 2,4-D or NAA was added to a medium containing Kn and BAP (Gupta et al., 1981, 1980). Addition of low levels of auxins (IAA or NAA, 0.05-0.1 mg/l) did not affect the growth and proliferation of shoots significantly in Quercus robur L. and Tilia cordata Mill. (Chalupa, 1984). In case of Halesia carolina, addition of NAA together with BAP, reduced the number of shoots formed alone with BAP (Brand and Lineberger, 1986).

However, there are reports of significant effect of auxin-cytokinin balance on the shoot formation. In kiwifruit, the highest number of shoots were formed with 2 mg/l BAP and 0.06 mg/l IBA (Monette, 1986). All combinations of BAP and NAA tested, induced axillary shoot proliferation on Euphorbia antisyphilitica explants (Jakobek et al., 1986). Buds of Carica papaya L. survived and grew better at Kn (50 µM) in combination with all levels of NAA (Rajeevan and Pandey, 1986). In Dalbergia sissoo, the best plantlet formation was obtained using a combination of Kn (1.0 mg/l) with IAA (0.5 mg/l) (Datta et al., 1983).

In addition to the reports mentioned above, there are other results too (Anderson, 1975; Hasegawa, 1979; Lane,

1979) which are similar to those obtained with Commiphora wightii. These indicate that the addition of low concentrations of auxins to the proliferation medium is of no benefit, and in some cases even deterious. It has been suggested that the addition of auxin (particularly NAA) to the medium strengthens apical dominance at the expense of shoot proliferation (Hildebrandt and Harney, 1983).

The results (Chapter III-3.b) also reveal that addition of activated charcoal (AC) was beneficial for the expansion of leaves. The beneficial effect of AC was also observed further in elongation and rooting of the shoots.

In Casuarina equisetifolia, the addition of BAP to MS medium containing NAA, markedly affected bud initiation, however, in the absence of AC (0.2%), no bud development, nor shoot elongation was observed (Duhoux et al., 1986).

Enhancement in the frequency of androgenesis with the use of AC has been observed in many plants (Anagnostakis, 1974; 1982; Wenzel et al., 1977; Sopory et al., 1978; Johansson and Eriksson, 1977).

Production of bulblets of Muscari armeniacum was enhanced by addition of AC in the medium (Peck and Cumming, 1986).

Weatherhead et al., 1978, believed that androgenic enhancement in tobacco is because of absorption of 5-hydroxymethyl-furfural by the AC. Charcoal is thought to

remove the inhibitory substances that may be present in the agar medium or originate from the explant itself. However, it is more probable that the level of growth substances (both endogenous and exogenous) is regulated by adsorption into charcoal (Bajaj, 1983).

To avoid the premature leaf drop of the in vitro grown shoots as mentioned in the Chapter III-3.c glutamine, adenine and phloroglucinol were tried at different concentrations. Of these only glutamine at 100 mg/l was found beneficial to control the premature leaf drop. Adenine had no effect and phloroglucinol at higher concentrations was observed inhibitory to shoot growth.

Similar observation in Leucaena leucocephala (Dhawan, and Bhojwani, 1985) reveal that though glutamine gave the best response, adenine also reduced leaf drop, but it was slightly toxic for shoot growth. In case of Tilia cordata Mill. (Chalupa, 1984), besides adenine sulfate (20 mg/l) and glutamine (10 mg/l), glycine (5 mg/l) was found essential for rapid shoot growth and multiple shoot formation. In Carob, incorporation of phloroglucinol (PG) (162 mg/l) enhanced general growth and leaf expansion of shoots. Presence of PG also induced regeneration of many shoots per culture. However, long term exposure to higher doses of PG was inhibitory to the culture (Thomas and Mehta, 1982). The phenolic compounds are known to participate in plant growth

regulation (Stenlid, 1968; Kefeli and Kutacek, 1977). It has been demonstrated that phloridzin, the 2'-glucoside of phloretic acid and its degradative products, phloroglucinol and phloretic acid can significantly enhance the growth of both shoots and roots of apple rootstocks cultured in vitro (Jones, 1979; James and Thurbon, 1979). On the contrary, results presented in this experiment reveal that addition of PG was of no benefit and had adverse effect on in vitro growth of shoots. Similar results have been reported for apple (Zimmerman, 1983; 1984 and Zimmerman and Broome, 1981) and for sour cherry (Jones and Hopgood; 1979 and Snir, 1983). PG has been reported to increase the level of auxin by protecting it from degradation (James and Thurbon, 1981). So malformation and callusing was observed during root formation in media containing both PG and auxin in guava shoot cultures (Amin and Jaiswal, 1987). The contradictory result about effectiveness of PG on different tree species may be explained by the difference between species and/or clones, and by the physiological state of material e.g. age, preconditioning and length of exposure to PG (Zimmerman, 1983). The specificity of these compounds in growth promotion has however remained controversial.

Beneficial effect of Thiamine HCl in forest and fruit trees has been reported by some of the workers (Monette, 1986; Lee and Thomas, 1985; Thomas, 1982). In Eucalyptus

grandis L. shoot proliferation rates increased with excess thiamine HCl (4 mg/l) in the medium (Rao and Venkateswara, 1985). The beneficial effect of thiamine HCl was also observed in Commiphora wightii shoot cultures (Chapter III-3.c.ii). Therefore, 100 mg/l glutamine and 10.0 mg/l thiamine HCl were routinely added in the maintenance medium.

The factors tried for the elongation of shoot cultures were addition of GA_3 (0.1 to 4.0 mg/l), state of medium with and without AC and reduced levels of cytokinins (Chapter III-3.d.i). No beneficial effect was observed with GA_3, on the contrary the cultures became weak at the higher concentrations of GA_3. Perez et al. (1985) had similarly observed that addition of GA_3 always resulted in thin and bended shoots in Corylus avellana L.. On the other hand, GA_3 has been proved effective in certain tree species. A brief treatment of cultures for a week with GA_3 resulted in rapid elongation of E. grandis shoots (Rao and Venkateswara, 1985). Lakshmi Sita and Shobha Rani (1985) also treated shoots of E. grandis L. with GA_3 (1 mg/l) for elongation prior to rooting.

For clonal propagation through in vitro culture of forest tree species, most of the work has concentrated on establishing optimum inorganic salts, vitamins, growth regulators and other chemical requirements. However, factors

other than the chemical composition of the medium may affect the shoot proliferation. Liquid culture offers advantages over agar-solidified medium, such as eliminating possible inhibitory compounds from the agar used (Romberger and Tabor, 1971).

For *in vitro* proliferation of *Populus alba* and *P. grandidentata* (Chun *et al.*, 1986), the cultures grew better in liquid medium than agar-solidified medium. Similar results have been reported for apple rootstocks (Snir and Erez, 1980), apple and pear (Singha, 1982) and for *Picea abis* (Romberger and Tabor, 1971). This may be partly due to an increased nutrient availability and uptake by the shoots. Less growth and fewer shoots on agar solidified medium have been interpreted as partly due to a lower diffusion rate of molecules, including exuded explant metabolites through the medium (Stevenson and Harris, 1980), partly to unidentified agar-borne inhibitors (Romberger and Tabor, 1971) and partly due to a reduction of water availability to tissue growing on agar solidified medium (Stoltz, 1971). To obtain shoot elongation in *E. citriodora* and *E. camaldulensis* (Gupta *et al.*, 1981, 1983), it was found necessary to transfer the cultures to shake flasks containing liquid medium of the same composition and incubate on a rotary shaker.

In the present investigations, the cultures in liquid

medium (either submerged or rotated), turned brown and died. Although, in stationary liquid culture in tubes supported on filter paper bridge, the shoots elongated, but exhibited vitrification symptoms as glassiness or water logging of the tissue. Most of the vitrification started after 2 weeks of culture in liquid medium. In liquid cultures shoot development was accompanied by callus formation (Chapter III-3.d.ii). Though liquid medium proved beneficial for some of the woody species, as mentioned earlier, nevertheless in vitro proliferation of most other woody species has been on agar based medium (Ahuja, 1983; Bonga, 1982; Thorpe and Biondi, 1984).

Addition of AC (0.3%) was found useful in preventing the vitrification effect in the cultures in stationary liquid medium and also established solid cultures in which liquid medium with charcoal was added.

Proliferating shoot cultures of Commiphora wightii maintained for longer period in medium containing 4 mg/l Kn and BAP showed suppressed shoot growth, but when these cultures were transferred to low Kn and BAP (0.4 mg/l of each) elongation of shoots was observed (Chapter III-3.d.iii). Hu and Wang (1983) reported that sometimes the elongation of shoots of micropropagation is inhibited by higher cytokinin level, thus an intermediate shoot elongation stage becomes necessary (George and Sherrington, 1984).

Similar results are reported with other forest and fruit trees. Blueberry shoots produced on a medium having 15 mg l^{-1} of N^6-γ, γ-dimethylallylamine purine showed sufficient elongation when transferred to a medium containing relatively low level (1-5 mg l^{-1}) of the cytokinin (Zimmerman and Broome, 1980 b). Elongation of laterally proliferated shoots of Prunus amygdalus too was satisfactory on MS medium lacking auxin but containing reduced BAP as the cytokinin (Rugini and Verma, 1982). Similar results were obtained by Perez et al. (1985). Transferring the shoot cultures of Corylus avellana L. from a primary explant to a fresh medium containing 25 μM BAP resulted in continued production of shoot buds; but growth of shoots was inhibited. Conversely on transferring these clusters to 0.5 μM or 2.5 μM BAP shoots elongated.

In carob tree cultures (Thomas and Mehta, 1982), high concentration of BAP was inhibitory for the growth, as the tissues turned hard and brittle. Further growth of the sprouted shoots was discouraged on the same medium and they were transferred to a medium with low cytokinins. Reduced levels of cytokinins were beneficial for the elongation of Sapium sebiferum shoots (Kotwal et al., 1983). In Stylidium coroniforme (McComb, 1985), the 1 μM level of BAP was optimal for the production of long, sturdy shoots suitable for root initiation. The fragile, tangled masses of

shoots produced at higher levels of BAP were difficult to handle with callus forming at the base of the shoots. Optimum elongation of shoots of E. torelliana was obtained on a medium with reduced levels of Kn (0.05 mg/l) and BAP (0.1 mg/l) (Gupta et al., 1983). In Euphorbia lathyris L. (Ripley and Preece, 1986) BAP at < 5 mgl^{-1} had little effect on explant shoot growth. As the level of BAP increased from 5 to 20 mg l^{-1} apical shoot growth decreased.

This inhibitory effect of cytokinins is expected since cytokinins are known to inhibit stem elongation and promote fasciation (Horgan, 1984; Wareing and Philips, 1981). High cytokinin levels initially stimulated axillary branching; however, continued exposure to high levels of cytokinins appeared inhibitory to further axillary shoot development.

Results presented in the Chapter III-3.e.i, reveal that none of the auxins (IAA, IBA, NAA) added singly at the conc tested induced root formation. Roots were induced with the combination of IAA and IBA at 1.0 and 5.0 mg l^{-1} respectively. However, prolonged auxin treatment caused callus formation. Moreover, the growth of the shoots was also affected.

For adventitious root formation IBA has been found superior over other auxins in many cases. Hutchinson (1981) has reported that IBA is the preferred auxin for root initiation. Jones and Hatfield (1976) found it superior to

IAA or NAA for *in vitro* rooting of apple shoots. Nevertheless, when IBA was used in combination with NAA, better rooting response was observed suggesting synergism between the auxins. Similar results have been reported by Ellyard (1981), Datta and Datta (1984) and Williams *et al.* (1984) wherein an equimolar conc of IBA + NAA or IBA + NOA gave improved rooting than either of the auxins alone. For *Quercus robur* L. and *Tilia cordata* high root initiation occurred when IBA 0.3 mg l^{-1} and NAA 0.1 mg l^{-1} were present in the rooting medium (Chalupa, 1984).

The growth of *C. wightii* roots was arrested in auxin supplemented medium. Development of roots was observed when the shoots with restrained roots were transferred to auxin-free medium after 24 and 48 h treatment with auxin. *Commiphora wightii* microcuttings rooted in the continuous presence of auxin in the medium showed inhibition of root development due to excessive formation of callus and/or malformation of roots. On transfer to auxin-free medium, after 24 h treatment in RIM (Root inducing medium) 60% rooting was observed in half strength MS medium containing AC (Chapter III-3.e.ii).

In several woody species, *in vitro* rooting can be improved if shoots are given a root inductive auxin treatment followed by transferral to auxin free media to allow for root development. Poor root development occurred

due to continued presence of auxin which was essential for the induction of root primordia.

Williams et al. (1984) reported that in vitro proliferated shoots of Dampiera diversifolia that failed to form roots on the medium with higher amount of IBA + NAA, produced cent per cent rooting on transfer to basal medium. With shoots produced by meristem culture in kiwifruit, the addition of IBA to the rooting medium encouraged the formation of large calli. More satisfactory results were obtained by dipping the shoots for only a few seconds in 2 mg l^{-1} IBA and placing them directly in the soil mix (Monette, 1986).

In Desert Milkweed (Lee and Thomas, 1985), a 48 h IBA pulsing treatment (preculturing) of cultures followed by a 4 week subculture on hormone-free medium greatly enhanced the rooting percentage as well as no. of roots per culture. The 335 D genotype of Liquidambar styraciflua (Sutter and Barker, 1985) shoot cuttings were placed on medium containing 1.0 mg l^{-1} IBA for 1 month during which they did not form roots; when transferred to medium lacking hormones the shoots started to form roots. When shoots of carob were exposed to the root inducing medium containing 1.0 mg l^{-1} IBA and 630 mg l^{-1} PG for 4 days and then transferred to a hormone free medium containing only 162 mg l^{-1} PG, 30% cultures formed roots. Similar results were obtained with M-26 apple root stock cultures. Further, high levels of IBA and PG

could induce rhizogenesis, but their continued presence limited subsequent root development and promoted callus formation at the cut ends of both carob and apple cultures (Thomas and Mehta, 1982).

The results discussed above may be due to dilution of the otherwise supraoptimal levels of applied auxins. However, it is suggested that auxin levels which promote root initiation may be unsuitable or even inhibitory for its further growth and development, and hence transfer of cultures to auxin-free medium after root induction would be beneficial (Torrey, 1956; Mohammed and Erikson, 1974; Mitsuhashi Kato et al., 1978). The inhibitory effect of IBA on root development is well known (Went and Thimann, 1937; Thimann, 1977) and the presence of the auxin for the entire root formation period could account for the significantly reduced number of roots produced in that treatment.

Complete dark period during root initiation phase (in RIM) was found to promote the rooting frequency in Commiphora wightii (Chapter III-3.e.ii). Hammerschlag (1982) stated that a 2 week dark period was essential for maximum rooting of Calita plum in vitro. Apple miorocuttings also showed increased rooting in darkness (Welander, 1983). Reasons for the enhanced rooting of stem cuttings under reduced irradiance or in darkness are not yet clear; however, it has been shown that levels of certain natural growth

inhibitors are lower in plant tissue grown in dark than that grown in light (Eliasson, 1971; Tillberg, 1974). The reduced levels of inhibitors may promote rooting under low light or dark condition. Absence of interferring effect of light on the root promoting action of endogenous auxin may also cause increased rooting in dark (Hartmann and Kester, 1986).

In the present study, half strength MS medium was found to be more suitable than White's medium. Moreover, the frequency of root formation was further increased when AC (0.3%) was incorporated in MS half strength medium (Chapter III-3.e.ii).

In Eucalyptus grandis (Rao and Venkateswara, 1985) the regenerated shoots formed roots in half strength MS medium. Preliminary experiments showed that rooting in Euphorbia lathyris (Ripley and Preece, 1986) was poor in full strength MS medium with IBA. The beneficial effect of AC is in accord with those of Snir and Erez (1980), and Cheema and Sharma (1983), where 100% rooting and a promotive influence of AC on root growth has been observed in apple root stocks. Anderson (1980) and Damiano (1980) have also reported the beneficial effects of AC on rooting of raspberries and strawberry respectively. AC was observed to play an important role in in vitro grown shoots from mature trees of E. tereticornis, E. globulus, E. torelliana and E. camaldulensis (Mascarenhas et al., 1982; Gupta et al., 1983).

In light of the experiments conducted on juvenile material, experiments were designed for the elite clones identified on the basis of chemical investigation.

Clonal propagation of elite clunes :

It is fairly well documented that *in vitro* establishment of woody plants can be influenced by the annual growth cycle of the tree and by the season during which explants are taken from the field-grown plants (Murashige, 1978; Skirvin, 1981; Hu and Wang, 1983; Hammerschlag, 1986). In *Commiphora wightii* maximum response with minimum contamination was obtained from the explants collected during April to June (Chapter III-4.a.). Similar effects of season on bud sprouting and explant contamination have also been reported for other trees like *Tectona grandis* (Gupta *et al.*, 1980), apple (Hutchinson, 1984), Sweetgum (Sutter and Barker, 1985) and guava (Jaiswal and Amin, 1987).

Shoot proliferation potential of *Halesia* explants, when correlated with shoot growth, was found to be greatest just after bud break, depressed during rapid shoot elongation and increased when elongation rates were slowed (Brand and Lineberger, 1986). Similarly, *Picea sitchensis* buds or young shoots taken at various times of the year were shown to contain the greatest organogenic potential at bud break, but lost this capacity when an advanced state of shoot elongation had been reached (Selby and Harvey, 1985).

Organogenic potential of Abies balsamea buds was also shown to be at its maximum when shoot growth had just initiated (Bonga, 1981).

Hu and Wang (1983) mentioned that in perennial species best results may be expected from the explants which were dissected at the end of dormancy period or prior to or during annual growth flush. It is likely that a balance of endogenous growth regulator levels is maintained during active growth period that causes the explants to respond favourably to the applied exogenous growth regulators. Seasonal changes in endogenous growth hormone type and concentration are known to occur in trees (Alvim et al., 1976; Dunberg, 1976; Junttila, 1982; Van Staden and Davey, 1981). The progression of shoot growth during spring flushing is likely to be controlled, at least in part, by hormonal fluctuations. Internal changes in a tree's hormonal status, which leads to changes in shoot growth, may also dictate the type of growth an explant undergoes when placed in vitro. In vitro development and organogenic potential of explants in various physiological states have been shown to be influenced by the levels of endogenous growth regulators (Cassells, 1979; Cassells et al., 1982; Heide, 1968).

In Salix pentandra increase in gibberellin like substances were found to accompany rapid shoot elongation during shoot flushing (Junttila, 1982). Both gibberellin-like

substances and IAA increased in Picea abis during the period of rapid shoot elongation (Dunbery, 1976).

In the present studies the buds collected from mature trees growing wild in forest offered serious problems for getting sterile cultures. After making numerous attempts, it was possible to obtain 90% sterile cultures by treatment with nystatin and streptomycin (Chapter II-3.a.2).

Many researchers working with adult material faced similar difficulties. Ripley and Preece (1986) found that Euphorbia lathyris shoot tips were not sensitive to rather prolonged exposure to sodium hypochlorite (NaClO) disinfectant. Contamination rates were high when disinfection time was less than 15 min. Contamination rates were low and no harmful effects were apparent after a 20 minute exposure. An improved surface sterilization method was used by Gupta and Durzan (1985) to disinfect the shoots of Douglas-fir and sugar pine. In Juglans nigra (Heile-Sudholt et al., 1986) when the etiolated axillary shoots derived from the mature tree explants were transferred for rooting, the microshoots exuded a milky white contaminant into the medium and completely covered surface of the agar. This was assumed to be an internal contaminant, which led to failure in establishing mature explants in vitro.

In the present study (Chapter III-4, b and c) only the

citriodora and E. torelliana and E. camaldulensis (Gupta et al., 1981; 1983), addition of biotin (0.1 mg l^{-1}) and ca-pantothenate (0.1 mg l^{-1}) proved beneficial for healthy shoot development. In Leucaena leucocephala maximum no. of shoots was obtained in medium containing both Kn and BAP. This effect was further enhanced when calcium-pantothenate and biotin (0.1 mg l^{-1} each) were supplemented to the medium (Kulkarni et al., 1984).

For elongation of shoots derived from mature explants, the same conditions i.e. solid medium containing reduced levels of Kn and BAP (0.4 mg l^{-1} of each), AC 0.5%, glutamine 100 mg l^{-1} and thiamine HCl 10.0 mg l^{-1}, used for the juvenile material also proved satisfactory with the only exception of addition of folic acid and biotin. Glutamine 100 mg l^{-1}, which was required in the maintenance medium, was not found to be essential for the further growth. It was, therefore, omitted from the elongation medium (Chapter III-4·e).

In the present study, the rooting response of shoots derived from mature explants was found to be similar to that of juvenile explants with a treatment of IAA and IBA (Both at 1.0 mg l^{-1}) for 24 and 48 h. They also rooted when treated with IAA and IBA at 5.0 mg l^{-1} each. An improved root system with laterals was developed when the AC level was raised from 0.3 to 0.5%. It was further observed that AC hastened the rooting response (Chapter III-4·f).

nodal explants with axillary buds gave rise to shoots, while the terminal and isolated axillary buds turned brown and died. Subsequently, it was further observed that the first nodal explant (unopened axillary bud 1.0 to 1.5 mm) exhibited 85% induction of bud burst when cultured on 8th day after excision of the terminal bud.

The choice of explant for micropropagation should be such that it gives maximum number of shoots when inoculated on a suitable medium. The age, size, source and type of explant influence this response (Skirvin, 1981). Apical buds, axillary buds and nodal explants are mainly used for shoot proliferation (Hutchinson, 1981), although stem, roots and leaves have also been utilised. Different organs respond to ingredients in the medium in different ways.

Zimmerman and Broome (1980 a) have reported the use of dormant axillary buds and meristems from one-year old shoots for apple cultivars. Only the nodal segments taken from mature trees of two pomegranate varieties could be successfully cultured and propagated (Iyer, 1984). Rapid clonal propagation of guava was obtained through <u>in vitro</u> culture of nodal explants (Amin and Jaiswal, 1987).

Nodal cultures of angiosperms are potentially valuable both from the applied and the theoretical points of view. On one hand, they offer single-bud starting material for the

precise study of shoot multiplication rates in micropropagation of certain species (George and Sherrington, 1984). On the other hand, they allow direct testing of the effects of a variety of media constituents on lateral shoot development, uncomplicated by apical dominance or other correlative influences (Peterson and Fletcher, 1975; Scorza et al., 1984). The added advantage of using nodal explants is that the shoot apical portions can be rooted for plantlet production and at the same time, culture can be maintained with basal nodal segments (Amin and Jaiswal, 1987).

The greater responsiveness of first nodal explants of Commiphora wightii over shoot tip and other nodal explants on 8th day can be attributed to the absence of apical dominance and presence of axillary buds at a more advanced stage of development.

In Liquidambar styraciflua (Sutter and Barker, 1985), shoot tips were excised from actively growing and dormant shoots and dormant buds were excised from both dormant and actively growing shoots. A lower percentage of buds (dormant shoots) survived than did buds from actively growing shoot tips. In apple rootstock KSC-3, even a relatively low dose of BAP (0.01 mg l^{-1}) stimulated some bud development in KSC-3 nodes. Such variation may be a reflection of varying innate 'sensitivity' or 'potential' of the different buds used in a particular treatment; this in turn, might be related to

initial bud position on the stem (Hicks and Nair, 1986). Notably, axillary buds of Rosa hybrida responded differently to BAP in vitro according to their position on individual lateral (donor) branches (Bressan et al., 1982). In their experiments, the most rapidly growing buds were from the middle of the stem.

Buds obviously do not require exogenous cytokinin, over the entire culture period, as seen in the transfers to less cytokinin media. One might speculate that exogenous cytokinin acts as a trigger to initiate development and that subsequently buds are relatively autonomous for the remainder of the culture.

Results presented in the Chapter III-4.d reveal that the nodal explants with dormant axillary buds derived from elite clones responded in similar way as the juvenile material. Multiple shoots (2 to 3 shoots per explant) were obtained in 80% of the cultures when Kn and BAP were incorporated in the medium at higher concentrations (4.0 mg l^{-1} of each). However, addition of glutamine, thiamine HCl and AC (0.1%) in the medium did not prevent the premature leaf drop. Increasing the level of AC (0.5%) and incorporation of folic acid (0.1 mg l^{-1}) and biotin (0.1 mg l^{-1}) were found beneficial for the growth of shoot culture.

For the shoots derived from mature trees of Eucalyptus

In Tectona grandis (Gupta et al., 1980), a lower conc of auxins (IAA, IBA and IPA, 0.1 mg l^{-1} each) was optimum for root induction in juvenile explants; whereas higher conc of these auxins (IBA, IPA and IAA at 2.5, 2.0 and 2.5 mg l^{-1} respectively) was necessary for the induction of roots in the mature explants. For root induction of shoots from mature trees of Dendrocalamus strictus, IBA at 10 times higher level for 96 h was required as compared to the seedling shoots which rooted by treatment with 0.1 ppm IBA for 48 h. Moreover, AC too was found essential for root initiation from mature shoots of D. strictus (Nadgir et al., 1984).

Results presented in the Chapter III-4.g reveal that the rooted plantlets derived from mature explants failed to elongate in the rooting medium. When different salt concs were tried, the shoots elongated in half strength of Wood and Broun's medium (WB) (1961) with reduced levels of Kn and BAP, without inhibiting root system. Sturdy plantlets with well developed root system were obtained on this medium.

Wood and Braun's medium (1961) is modified White's medium, but contains much higher level of phosphate, sodium nitrate and ammonium sulphate. Though the other salt formulation is lower than in MS, phosphate and sodium level is much higher than in MS. Once rooted, the plantlets probably require dilute salt formulation (1/2 strength WB

medium) but with high phosphate and sodium level. Reduced levels of Kn and BAP (0.4 mg l^{-1}) proved beneficial. The conc of KNO_3 is very low in WB medium as compared to MS. It is possible that some component of MS salts is toxic for elongation of Commiphora wightii shoots, KNO_3 being a possibility, since WB medium contains a lower level of KNO_3.

Similarly, for Halesia, woody plant medium (WPM, Lloyd and McCown, 1980) was found more suitable than MS medium (Brand and Lineberger, 1986). Liquidambar styraciflua browned and died on MS medium, but was successfully grown on Blaydes' salts (Sommer and Caldas, 1981) and WPM (Sutter and Barker, 1985) since WPM lacked KNO_3.

In E. citriodora, the rooted shoots derived from mature explants failed to survive on White's medium, the leaves gradually turned yellow and dropped off. If the plantlets were transferred within two weeks, just after the emergence of the roots to liquid MS medium with biotin and ca-pantothenate, the yellowish leaves turned green and well developed plants were formed (Gupta et al., 1981). In Carica papaya L. (Rajeevan and Pandey, 1986), IBA produced a well developed root system; but the shoot system did not develop properly. The shoots remained stunted, resulting in abnormal plantlets. The failure of shoots to elongate even after rooting seemed to indicate the possibility that the lateral buds were originally under quite strong control of apical

dominance. This inhibitory effect might have been carried by the shoots originating from lateral buds and thus behaving fundamentally like an inhibited bud even after rooting.

Micropropagated Commiphora wightii plants, once established in soil, showed vigorous and uniform growth throughout the observation period of two years (Chapter III-4.h).

Acclimated plantlets appeared normal in all respects and grew rapidly under greenhouse conditions. No morphological abnormalities have been observed in the tissue culture raised plants.

Direct transplantation in potting mix was unsuccessful. However, with the method involving a pretransplantation hardening stage (Chapter II-5), 60% plants survived. Some of these plants are now growing in the garden beds. The plants derived from the adult material are found to be sturdier than the plants of juvenile origin.

In woody species, the frequency of regeneration in vitro generally declines with the age of the explant source. With E. grandis the nodal explants from juvenile as well as adult trees responded alike for shoot induction. However, shoots induced from juvenile material were more amenable to rooting than those from mature trees (Rao and Venkateswara, 1985).

Results of the field trials demonstrated the

feasibility of the application of tissue culture for raising elite plants of Commiphora wightii. Results with tissue culture raised plants produced from the high yielding E. citriodora trees (Gupta and Mascarenhas, 1982) have equivocally demonstrated that the high oil character is retained in tissue culture raised plants even when grown under different agroclimatic conditions.

The multiplication rate in the present studies is not sufficiently large to become commercially viable. However, these results provide a basis for further research on improvement of clonal propagation of Commiphora wightii which yields a minor forest product of economic importance. Moreover, it also provides a source of income for the tribals.

VI-2. EFFECT OF CULTURAL PARAMETERS ON GROWTH OF COMMIPHORA WIGHTII CELL CULTURES

Success in the technology and application of in vitro methods is largely due to a better understanding of the nutritional requirements of cultured cells and tissues (Street, 1977). General nutritional milieu and explant origin are the two factors which most frequently determine the success of cell cultures. The nutritional milieu consists of essential and optional components. The essential nutrients

consist of inorganic salts, a carbon and energy source, vitamins and growth regulators. Other components, including organic nitrogen components, organic acids and complex substances are optional choice of media (Gamborg and Skyluk, 1981).

A wide variety of salt mixtures have been reported (Conger, 1981). Several have been tested with callus and cell cultures of a wide variety of plant species. The choice depends on the plant species and to a degree upon the intended use of the culture.

Commiphora wightii callus was originally initiated from stem and leaf explants onto MS, White's and B5 mineral formulations, supplemented with 2,4-D and Kn (0.1 mg l^{-1} of each). Of these MS medium supported the maximum growth (Chapter IV-1.a).

In the case of Phaseolus vulgaris culture, the yield of callus was about the same whether the mineral salts were added at the same level as in MS medium or when diluted to one-half of that strength. Thus, the main effect of the mineral salt mixture is due to composition rather than the high concs. (Liau and Boll, 1970). The explants produced callus first at the cut surfaces. As a result of injury the local level of auxin presumably rises and quiescent cells are stimulated to divide rapidly, producing a mass of cells. This may be due to the fact that the cells at the cut

surfaces are exposed to an excessive supply of nutritive substances as compared with the cells of the adjoining intact regions of the explant (Mitra et al., 1965).

We also observed that larger explants (leaf discs 5 mm diameter, stem explants 5-10 mm) callused more frequently and easily than the smaller ones. The smaller explants probably have lower levels of endogenous growth substances as a result of loss from small pieces. Gautheret (1955) conducted studies on the effect of size of explant on the growth of carrot tissue and found that the larger explants grew in the medium without auxin, whereas smaller ones required the presence of IAA in the medium. Mehra and Mehra (1974) suggested that possibly important metabolites were lost from the cells when the stem explant size was reduced.

Studies on the morphogenetic potentiality of tissue indicated that the leaf explants were more responsive to the auxin treatment than stem explants (Chapter IV-1.b; III-5.b). Of the four auxins tested, NAA was found more effective for inducing rhizogenesis followed by IAA and IBA, whereas 2,4-D was the least effective.

Leaf explants have been found to be more responsive either to organogenesis or embryogenesis in a number of species e.g. Populus tremuloides (Noh and Minocha, 1986); Leucosceptrum canum sm (Pal et al., 1985); Ulmus X 'Pioneer' (Fink et al., 1986); Santalum album (Rao and Bapat, 1978).

Studies on nutrition in callus cultures :

Carbohydrate nutrition :

Since the inception of plant cell culture, carbohydrate nutrition has been an important area of study. Intact plants meet their energy requirements autotrophically via photosynthetic carbon fixation. In cultured plant cell cultures, the normal functions of chloroplasts are usually partially or totally blocked, hence necessiating the incorporation of carbohydrate into the nutrient medium. Carbohydrate requirement of callus cultures have been worked out by a no. of researchers (Hildebrandt and Ricker, 1953; Subbaiah et al., 1974; Nambiar, 1980; Ravishankar, 1980). Most of these studies have revealed the superiority of sucrose over other carbohydrates as a carbon source. Carbohydrate metabolism in cell and tissue cultures have been extensively reviewed (Maretzki, et al., 1974; Fowler, 1978). Sugars not only provide with carbon skeleton for the synthesis of varied compounds primary as well as secondary, but also constitute the source of energy for all the metabolic activities (Simpkins and Street, 1970).

Present studies with Commiphora wightii callus cultures also showed that sucrose (2%) is a better carbon source than the other sugars studied; however, glucose was also found to support good callus growth (Chapter IV-2.a; IV-2.d). With Gomphrena globosa callus cultures, Ruiz and Valadez (1985)

observed increases in dry weight of the tissue with increasing sucrose concentration. Sucrose at 2% concentration was observed to be optimum for Punica granatum callus cultures (Jaidka and Mehra, 1986).

Phytohormones :

The auxins and cytokinins are the two classes of compounds which are of special importance in plant tissue culture. Among the various auxins tried; 2,4-D at 0.2 mg l^{-1} and NAA at 1.0 mg l^{-1} induced healthy growth of callus cultures (Chapter IV-2.b). The results showed that the auxins supplied exogenously promoted growth at lower concentrations in general, whereas growth was suppressed at higher levels of auxin, suggesting that the regulatory mechanism of auxin was controlled by its level maintained endogenously. Similar results have been reported by Risser and White (1964), Mitchell and Gildow (1975). 2,4-D in general is a more effective auxin for callus induction.

Cytokinins are reported to have an additive effect on growth of tissue (Fox, 1969). Incorporation of Kn or Zeatin into the culture medium led to substantial enhancement in growth of a number of callus and suspension cultures (Steward, et al., 1969; Subbaih et al., 1974). This effect of cytokinins is mainly due to their effects on cell division, protein and nucleic acid metabolism (Skoog et al., 1967; Letham and Williams, 1969).

Of the three cytokinins tested, Kn supported the maximum growth of callus cultures of Commiphora wightii at 0.2 mg l^{-1} followed by BAP; the higher concentrations had inhibitory effect though (Chapter IV-2.c).

Hormone effects on protein synthesis are generally achieved through enhanced RNA synthesis, formation of polyribosomes or through an interaction with the t-RNA and ribosomes as in the case of cytokinins.

Hormones, apart from influencing the synthesis of enzyme proteins, directly or indirectly may also influence their ability. It is evident that enzymes concerned with all aspects of plant metabolism are influenced by the plant hormones. The effects may be both promotive or inhibitory, the extent depending on the higher conc being inhibitory in most cases. Both synthetic and degradative enzymes are affected. Apparently the totality of such effects determine the overall physiological response. Hormones may also modify the confirmation of enzyme proteins e.g. RNA polymerase (Sen, 1984).

The balance between the two hormones is important in controlling DNA synthesis and cell division. Torrey (1967) reported that Kn 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.

Vitamins :

Normal plants synthesize the vitamins required for growth and development. When cells of higher plants are grown in culture, some vitamins may become limiting.

It was observed in the present studies that the vitamins viz. niacin, thiamine and pyridoxine did not enhance callus growth when added singly; however, when added in combination, healthy growth of callus was obtained (Chapter IV-2.e).

Risser and White (1964) found that spruce tumour tissue requires thiamine (1 mg l^{-1}), niacin (10 mg l^{-1}), ascorbic acid (10 mg l^{-1}), and inositol (100 mg l^{-1}) for continuous healthy growth of callus. Vit B_{12}, Ca-pantothenate, riboflavin, biotin, choline chloride and pyridoxine were also required for this callus. Been callus and suspension cultures required thiamine at higher concentration than the usual concentration of Linsmaier and Skoog's medium (Liau and Ball, 1970).

Inositol enhanced callus growth over a wide range from 20 to 400 mg l^{-1} with the optimum conc at 200 mg l^{-1} (Chapter IV-2.f), when the callus became friable.

There are similar reports of enhancement of callus growth (Kumar, 1974; Risser and White, 1964) with the incorporation of inositol.

Myo-inositol is a standard constituent of bacterial and

animal cell culture media. Most plant tissue culture media also contain myo-inositol. 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). Auxins have been reported to form conjugates with myo-inositol. Indole-3-acetyl-2'-myo-inositol has been isolated from several tissues. Inositol has been found to bind with more than one molecule of IAA and such conjugates also occur in nature. These complexes are highly active in tissue culture growth. In the complexed form, IAA is probably protected from oxidative degradation (Sen,1984).

Comparative studies between static and suspension cultures (Chapter IV-1.c; IV-3.c) clearly showed that growth of cells was appreciably higher and fast in liquid shake culture. The maximum growth (Fr. Wt. 10.4 gm, dry wt. 0.3 g) obtained on 16th day in suspension culture is 4.7 and 3.75 fold on fresh and dry wt. basis as compared with the static cultures. A high degree of friability of the ivory calluses grown on 2,4-D, Kn (0.2 mg l^{-1} of each) media made them particularly suitable for starting a suspension culture. The hormonal conc used for suspension culture (Kn and 2,4-D at 0.1 mg l^{-1}) is just the half as compared with the static cultures. This might be on account of efficient uptake of nutrients by cell suspension.

The dispersion of the callus tissues has been observed to be more dependent upon the composition of the nutrient rather than upon the agitation of the culture. Under appropriate nutritive conditions certain callus cultures become more friable to give a suspension containing free cells and cell aggregates of a few to several hundred cells (Street, 1966).

In Acacia senegal, only a few hours of vigorous agitation of calluses, provided an efficient dissociation of the cells (Hustache et al., 1986). Aspects of growth in suspension culture of Phaseolus vulgaris L. and Linum usitatissimum (Mehta et al., 1967) and Parthenocissus tricuspidata, Acer pseudoplatanus (Henshaw et al., 1966) have been extensively studied.

VI-3 EFFECT OF CULTURAL PARAMETERS ON PRODUCTION OF Δ^{β}-C-3 STEROLS AND GUGGULSTERONES

Isolation of several steroidal constituents from the gum resin of Commiphora wightii has been reported (Patil et al., 1972) and two of these (Z- and E-guggulsterone) have since been shown to possess significant hypocholesterolemic activity (Nityanand and Kapoor, 1971; Malhotra et al., 1970).

Biogenic pattern

There is sufficient evidence that both in mammalian tissues (Dague et al., 1979) and in plants (Haftmann, 1975), the catabolism of cholesterol proceeds by either of the two major pathways shown in Fig. VI-1.

Cholesterol is first monohydroxylated either at C-20 or C-22 followed by introduction of a second hydroxyl group at the position not previously attacked. The dihydroxy-cholesterol is then cleaved to give pregnenolone.

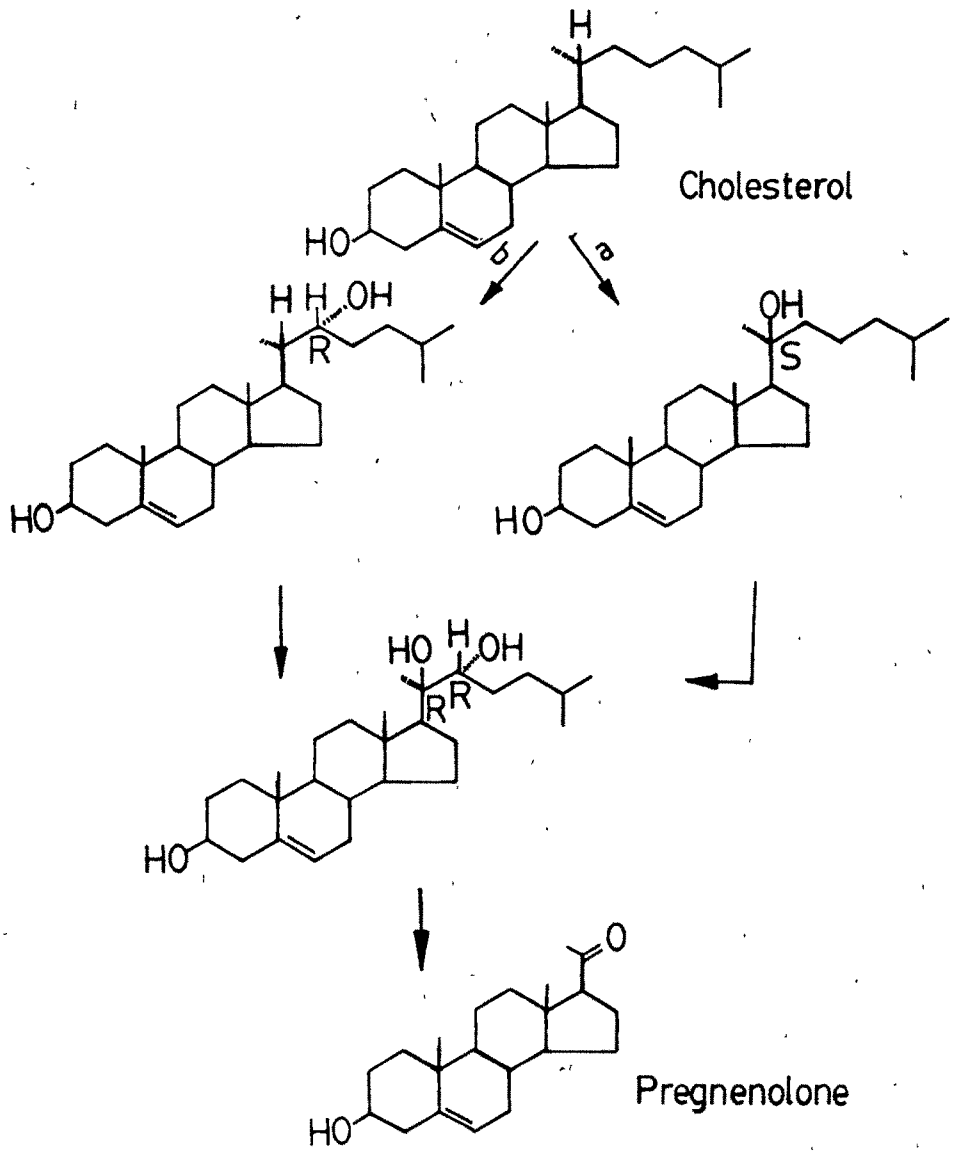
Cleavage of the cholesterol side chain is one of the key reactions leading to the biosynthesis of the steroids. Activity of this enzyme is localized in the mitochondrial fraction of the tissues.

The steroids from guggul provide an unique example of occurrence of cholesterol and each of the key intermediates according to pathway 'a', but with additional hydroxylation at C-16 in such a catabolic sequence in the plant tissue (Fig. VI-2)

Cholesterol ⟶ Guggulsterol II ⟶ Guggulsterol III

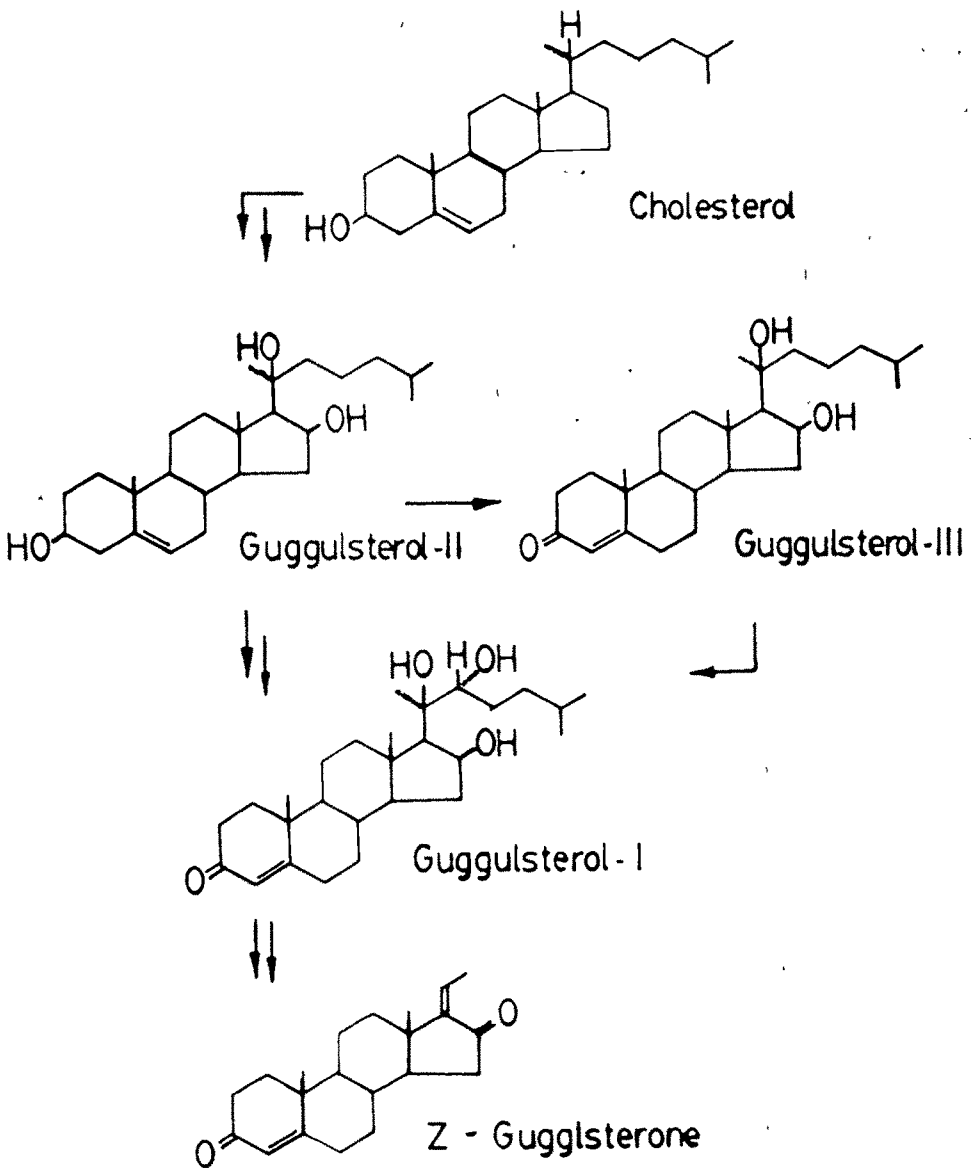
guggulsterol I ⟶ Z guggulsterone

(Bajaj and Dev, 1982).



CATABOLISM OF CHOLESTEROL TO PREGNENOLONE

Fig. VI-1



BIOGENESIS OF GUGGULU STEROIDS

Fig.VI-2

Fig. VI-2 Biogenesis of guggulu steroids.

Phytosterols

All plant tissues have the potential to synthesize sterols (Evans, 1974), but the rate of synthesis depends upon the type and age of the tissue (Geuns, 1975) and on environmental factors (Davis and Finker, 1972). It is becoming increasingly evident that all plants contain sterols of some kind and that they are vitally important cell constituents. One of the important functions of sterols in plants is to serve as precursors for the synthesis of other steroids (Heftmann, 1975). Nes and McKean (1977) have discussed the biosynthesis of sterols in plants and they have also pointed out some variations in the biosynthetic pathways and some differences in the nature of sterols produced by different plant species. These products or intermediates are utilized in various ways by various plants to produce more or less characteristic steroids.

The sterols most common in higher plants are sitosterol, stigmasterol and campesterol (Stoll and Jucker, 1955). Cholesterol, which for many years was considered to occur only in animals has now been found in many plant tissues (Grunwald, 1975). It also occupies a key position in the biosynthesis of other steroids (Haftmann, 1975).

Progestagens

Progestagens are steroids with 21 carbon atoms and a number of these steroids have been isolated from various plants (Gawienowski and Gibb, 1968). Generally, of all the steroids with C-21 carbon atoms, pregnenolone and progesterone receive the greatest attention, partly because they are hormones in animals, but also because they are precursors to the cardenolides which are found in plants. The 21 carbon steroids are derived from sterols by oxidative cleavage of the C-17 side chain (Pilgrim, 1972). The conversion of cholesterol to pregnenolone has been demonstrated with a number of plants (Bennett and Haftmann, 1966; Sauer et al., 1967; Caspi et al., 1966). Intermediates in the degradation process are 22 α-hydroxycholesterol and 20α, 22 β dihydroxycholesterol. 22β-hydroxycholesterol might also be an intermediate (Burstein et al., 1970) as already stated. But not all plants are capable of converting this compound (Stohs and El-Olemy, 1971). Furthermore, not all plants contain cholesterol and therefore the question arises whether other sterols, especially the common 29 carbon sterols, can be converted to pregnenolone. The answer was affirmative when experiments with (3-^{14}C)- sitosterol were carried out (Bennett et al., 1969).

The degradation of cholesterol to pregnenolone has been observed in vivo not only in Holarrhena floribunda (Bennett

and Haftmann, 1965), but also in Digitalis lanata (Caspi and Lewis, 1967) and in vitro in tissue culture of D. purpurea, D. lutea and Nicotiana tabacum (Graves and Smith, 1967). Other sterols may undergo analogous degradations in plants. It was found that sitosterol is similarly converted to progesterone by Digitalis lanata plants (Bennett et al.,1969).

As mentioned earlier (Chapter I-3), the active principle (-Z and -E guggulsterone was found 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. The callus as well as suspension cultures of Commiphora wightii contain β-sitosterol, cholesterol and campesterol (Chapter V-1.a.2). Since as mentioned above, other sterols also undergo degradation analogous to cholesterol, β-C-3 sterols together were considered as precursors of these compounds and experiments were planned to raise the content of β-C-3 sterols in cell cultures.

Similar approach has been reported earlier in the case of Catharanthus roseus. The antitumor alkaloids, vincristine and vinblastine (Kurz et al., 1980) found in very low concentration in plants of Catharanthus roseus. However, the precursors of vinblastine, vindolinine and catharanthine are produced in plant cell suspension cultures, and these can be joined by chemical synthesis to form vinblastine (Lounasmaa

and Nemes, 1982). In this way, it might be possible by combining biotechnology and chemistry, to obtain compounds that are not produced by plant cell suspensions.

Factors affecting secondary metabolite accumulations in cell cultures :

Improvement in the secondary metabolite productivity of plant cell cultures can be made by taking explants from parent plants which accumulate high levels of a particular secondary metabolite. However, within a plant the level of secondary metabolites again varies in the different parts and so the plant tissue part used to start with the culture may affect the product formation.

The levels of the four major sterols sitosterol, cholesterol, campesterol and stigmasterol vary during floral development in Lolium lemulentum and was observed to be maximum in the shoot apex (Garg and Paleg, 1986).

Changes in the sterol composition during developmental processes such as seed germination (Bush and Grunwald, 1972); hypocotyl elongation (Geuns, 1974); potato tuber formation (Hartmann and Benveniste, 1974) have been studied.

There is evidence that cultures established from a single plant differ in yield of secondary products. This has been shown with tobacco by Kinnersley and Dougall (1980)

using primary explants from different leaves and stem pith from different internodes of individual plant. Bhatt and Bhatt (1984 a) observed that the distribution of sterols in Solanum nigrum leaves on dry weight basis varied in the young and mature leaves. Moreover, they also observed that the in vitro cultures significantly altered in the sterol composition and level of initial leaf explants. Similar observations have been made by Brain and Lockwood (1954) in Trigonella foenumgraecum callus cultures initiated from germinated seeds. Galanes et al. (1984) reported that diosgenin was absent in the callus and cell suspension cultures of Solanum aviculare. This might be attributed to the explant source. The explant was selected arbitrarily and there was the possibility that the explants chosen were not capable of synthesizing diosgenin in appreciable quantities.

The results in (Chapter V-1.a. clearly indicated that the total sterols are accumulated in comparatively more quantity in the stem than in the leaves. So also the callus derived from them.

The kinetics of total sterol content suggested that on per cent wise, the total sterol content drops during lag phase, increases slowly during exponential and stationary phases (Chapter V-1.b).

We have observed that the stem derived callus contained more quantity of β-C-3 sterols than the leaf derived callus as mentioned earlier. This prompted us to carry out further detailed studies on the callus initiated from stem explants. We also observed that growth of cells was faster in the suspension cultures than the callus cultures. This might most probably be due to the greater aeration, easy availability of the nutrients and a larger cell surface area for absorption. So, the further experiments were carried out using suspension culture of stem derived callus.

The results obtained in the experiment V-2.a indicated that MS standard nitrate containing medium and the x 0.5 level of nitrate (NH_4NO_3 10 mM and KNO_3 10.0 mM) supported growth of cells, but however, the sterol content dropped.

Nitrogen plays an important role in secondary metabolism. Nambiar (1980) observed no alkaloid production in the absence of exogenous nitrogen, whereas increasing concentration of nitrogen upto 840 mg l^{-1} induced the synthesis of alkaloids in Evolvulus alsinoides L. suspension cultures.

The catecholic amino acid 3-(3,4-dihydroxyphenyl)-L-amino(L-Dopa) production in nitrogen limited Mucuna pruriens cultures was strongly diminished by only lowering the nitrogen content in the medium to 50%; L-Dopa production falls to approximately 10% of the production in a control

culture (Wichers et al., 1985). De-Eknamkul and Ellis (1985) reported that the optimum concentration was 15 mM NO_3^- for both growth and rosmarinic acid formation, which indicated that concentration of NO_3^- in B5 medium was supraoptimal for cell growth and rosmarinic acid formation in Anchusa officinalis cell cultures.

The effect of nitrogen on steroid metabolism had been investigated by few researchers. Heble and Staba (1980) observed that the cells of Dioscorea deltoidea in the stationary phase from nitrogen depleted medium or medium with hydroxy urea contained low levels of sterols and diosgenin. Both NH_4^+ and NO_3^- were required at a certain ratio (KNO_3 1.9 g/l and NH_4NO_3 - 1.65 g/l) to enable diosgenin production in Dioscorea suspension culture (Rokem et al., 1985).

However, the enhanced total sterol content, we observed with the increased nitrate level was correlated with the reduced cell growth.

Studies carried out with Commiphora wightii suspension cultures to find out the effect of high concentration of inorganic phosphate revealed that at the X 1.5 phosphate level, both the cell growth and total sterol content was enhanced (Chapter V-2.b).

Phosphate is a wellknown modifier of secondary

metabolism in microorganisms (Martin, 1977). The production of secondary metabolites in microorganisms is commonly associated with slow growth and with low concentration of orthophosphate in the medium. This was also observed true in the case of higher plants, e.g. Nambiar (1980) observed that at the highest level of phosphate tested, alkaloid production was drastically reduced and was much below the control in Evolvulus cell cultures. Higher level of phosphate has also been found to strongly repress secondary metabolism in cell suspension cultures of Catharanthus roseus, Nicotiana tabacum and Peganum harmala (Sasse et al., 1982). However, in other cases, product formation was improved by increasing the phosphate concentration, e.g. the formation of anthraquinone in Morinda citrifolia (Zenk et al., 1975). Lowering the phosphate conc. resulted in a drop in both the final yields of biomass and of rosmarinic acid content in Anchusa cultures (De-Eknamkul and Ellis, 1985). Rokem et al. (1985) have reported that an increase in the phosphate conc. resulted in a marked increase in diosgenin when expressed both as % of dry weight and total conc.

Demain (1972) has proposed that phosphate inhibition or activation might involve regulation of energy charge.

It is well known that the biosynthesis of steroids proceeds through the formation of squalene. The metabolic route from mevalonic acid to isopentenyl pyrophosphate, a

precursor of squalene is indicated in Fig. VI-3. Mevalonic acid is first converted to mevalonic acid 5-phosphate by mevalonic kinase and subsequently to mevalonic acid 5-pyrophosphate. The latter substrate then undergoes a concentrated dehydration and decarboxylation, yielding isopentenyl pyrophosphate. This reaction, possibly, involves the formation of 3-phosphomevalonic acid-5-pyrophosphate as an intermediate. The conversion of isopentyl pyrophosphate to farnesyl pyrophosphate occurs via several successive condensations. It is clear that farnesyl pyrophosphate serves as the precursor in the formation of squalene (Mahler and Cordes, 1966).

Since, most of these reactions leading to the formation of squalene require ATP, the possibility of enhancement in the phytosterol or diosgenin production with orthophosphate can be explained.

Of all the carbohydrates tested, sucrose supported the growth of cells as well as sterol production followed by lactose and galactose (Chapter V-2.c). Studies on effect of different levels of sucrose, which proved the best source of carbon and energy, indicated that increasing sucrose levels upto 6% enhanced sterol production. However, 2% sucrose was optimum for growth of cells (Chapter V-2.d).

As stated earlier, carbohydrates not only provide the energy source, but also supply carbon skeleton for the

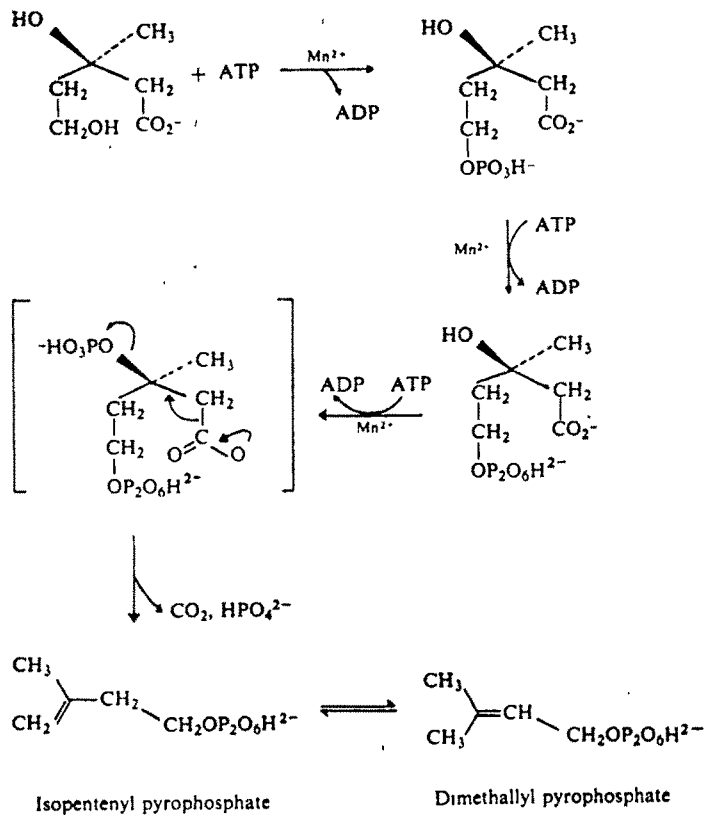


Fig. VI-3 Biosynthetic pathway from mevalonic acid to isopentenyl pyrophosphate.

synthesis of secondary metabolites in plants. The nature and conc of carbon source is thus important for product formation.

The effects of sucrose conc on the yield of secondary products have been examined in a number of plant cell cultures. The optimum conc seems to vary according to the plant species. The initial sucrose conc influenced the rate of synthesis and final conc of diosgenin in Dioscorea deltoidea cell cultures (Rokem et al., 1985). The highest diosgenin conc was obtained with 15 g l^{-1} sucrose whereas the highest productivity was obtained with 30 g l^{-1} sucrose. The highest sucrose conc increased anthocyanin yield in the carrot suspension culture, even at high inoculum desities (Ozeki and Komamine, 1985). In general, raising the initial sucrose levels leads to an increase in the secondary metabolite yields. Perhaps the effect of high initial level of sucrose is to raise the osmotic potential of media.

Although, sucrose is generally the most suitable carbon source for plant cell cultures, many cultures can assimilate other carbon sources, usually with lower efficiency. In Anchusa cell cultures, fructose was as effective for growth and rosmarinic acid formation as sucrose, whereas glucose was slightly inferior to sucrose and fructose, however, a 1:1 ratio of glucose to fructose also proved to be as effective as sucrose (De-Eknamkul and Ellis, 1985). Galactose was

observed to be the most suitable carbon source for quassin accumulation in Picrasma quassioides cell suspensions (Scragg and Allan, 1986).

Sucrose was, however, much superior to glucose and fructose for the formation of shikonin derivatives in Lithospermum erythrorhizon cultures (Mizukami et al., 1977).

The secondary metabolite production is influenced not only by the nature of auxins but their conc too. The total sterol content fluctuated at the different conc of auxins tested. Among the auxins studied, 2,4-D at 0.01 mg l^{-1} promoted the highest sterol content followed by NAA, IBA and IAA. However, at higher conc i.e. 1.0 mg l^{-1}, maximum sterol content was obtained with IAA followed by 2,4-D, NAA and IBA (Chapter V-2.e).

Earlier Khanna et al. (1975) had reported that in Trigonella foenum-graecum L. suspension cultures, NAA and GA singly showed decrease in diosgenin and other steroids (Gitogenin, Tigogenin, β-sitosterol, Stigmasterol) whereas tissues grown on media supplemented with IAA and kinetin singly, showed many-fold increase in sapogenin and steroids. However, in tissues grown on 2,4-D supplemented media, a reduction in the amount of diosgenin and other steroids was observed; but the conc of 2,4-D they used was above 1.0 mg l^{-1}. In Solanum mammosum callus cultures, Gunawan and Isnaeni (1986)

obtained good callus growth as well as β-C-3 sterol production with kinetin and 2,4-D.

Plant growth regulators are effective triggers of secondary metabolism *in vivo* (Böhm, 1980). Similarly, *in vitro*, both the quality and quantity of auxins initially present in the media or administered during the course of culture development have a marked effect on primary (Everett, *et al.*, 1981) and secondary metabolism (Gamborg *et al.*, 1971). Since the production of secondary metabolites in plant cell cultures is a function of both cell multiplication and division, growth regulators have a major role in determining the potential production of a given culture (Mantell and Smith, 1983). Heble *et al.* (1971) have shown that callus cultures of *S. xanthocarpum* are capable of producing solasodine when grown on a medium containing 2,4-D. The cultures also produce diosgenin and sitosterol. However, *S. xanthocarpum* cultures grown in the presence of IAA or IBA in the place of 2,4-D, produced no solasodine. Earlier, Furuya *et al.* (1971) observed that in callus cultures of *Nicotiana tabacum*, phytosterols and triterpenoids, but no alkaloids were formed in the presence of 2,4-D, while the reverse occurred in the presence of IAA.

In most studies, auxins have been applied to cultures in combination with cytokinins. The combined effects of auxins and cytokinins are difficult to assess particularly

since there is lack of data on relative endogenous levels of these growth regulators in the cultured cells; still, certain combinations are beneficial than others.

Brain and Lockwood (1954) observed varied effect of addition of cytokinin with respect to the conc of 2,4-D in the Trigonella foenum-graecum suspension cultures. With 0.1 mg l^{-1} 2,4-D, there was a concurrent increase both in the free sterols and dry wt upto 1.0 mg l^{-1} Kn and then decrease in free sterol and dry wt. upto 10 mg l^{-1} Kn conc. In the presence of 1.0 mg l^{-1} 2,4-D, however, both free sterol and dry wt. were suppressed with the addition of Kn and this effect was more marked with coconut water. However, maximum free sterol content was obtained at the lower conc of 2,4-D i.e. 0.1 mg l^{-1} 2,4-D with Kn 1.0 mg l^{-1}. In Dioscorea deltoidea suspension culture, 2,4-D 1.0 mg l^{-1} with Kn 0.2 mg l^{-1} promoted growth of cells; but the highest diosgenin content 1.0% of dry wt. was obtained with 0.1 mg l^{-1} 2,4-D and 0.2 mg l^{-1} Kn (Rokem et al., 1985).

We have observed that of the various combinations of 2,4-D and Kn tried, 2,4-D at 0.1 mg l^{-1} with Kn at 0.1 mg l^{-1} gave the maximum cell yield; whereas the maximum sterol content was obtained with 0.01 mg l^{-1} 2,4-D with 0.1 mg l^{-1} Kn (Chapter V-2.f). It was also observed that 2,4-D was more effective in influencing the sterol content than Kn. In Solanum aviculare cultures, reduction on both auxin and

cytokinin levels in the medium led to an increase in the steroid spectrum (cholesterol, stigmasterol, campesterol and β-sitosterol). By applying an auxin/kinin stepdown subculturing procedure, the same cell line was induced to accumulate a wider range of steroid constituents which included diosgenin and other steroidal alkaloids. It was also observed that the effect of auxin was more predominant than cytokinin on steroidal sapogenins and biomass in S. aviculare cultures (Mantell and Smith, 1983).

In the experiments conducted to study the effect of different levels of micro and macroelement salts, it was noticed that the macroelements i.e. $CaCl_2$, $MgSO_4$ and Na_2Fe EDTA had more pronounced effect than microelements. Reduced (X1.0) level of micro and macro elements was optimum for growth; whereas X 2.0 macro + X1.0 micro, and X2.0 macro + X2.0 micro elements slightly enhanced the sterol content (Chapter V-2.g). The effect of single macro or microelement was, however, not studied further, since this expt. did not exhibit any significant effect of the combined macro and micro elements.

While examining the effect of magnesium conc on diosgenin production by Dioscorea deltoidea cell cultures, Rokem et al. (1985) found that magnesium had no effect on diosgenin levels. $CaCl_2$ was observed to have strong effects on growth and rosmarinic acid production in Anchusa cultures. Cell growth and rosmarinic acid content of Anchusa cultures reached their

highest values at a Ca^{2+} conc at 0.25 mM, which is one-quarter than that in normal B5 medium (De-Eknamkul and Ellis, 1985).

White's medium contains 15 kinds of inorganic components, of which NO_3^-, Cu^{+2} and SO_4^{-2} demonstrated the strongest effects on the production of shikonin derivatives. The yield of shikonin derivatives increased with an increase in the conc of Cu^{+2}, but became constant when the conc exceeded 0.8 mM. The conc of Cu^{+2} had almost no effect at all on the cell growth. The yield of shikonin derivatives steeply rose with an increase in the conc of SO_4^{-2}, and the optimum conc was found to be 13.5 mM. Cell growth was slightly inhibited by SO_4^{-2}(Yamada and Fujita, 1983).

Attempts were made to elucidate the effect of some main precursors of sterols i.e. acetate, mavlonate on the sterol production. Results obtained in the present studies with Commiphora demonstrated that sodium mavlonate was utilized by the cell cultures more effectively for the production of sterols than sodium acetate. When cholesterol was added in the medium, it had an inhibitory effect on sterol production (Chapter V-2.h).

An exogenous supply of biosynthetic precursor to culture medium may increase the yield of the final product. The production of tropane alkaloids can be markedly increased by

the addition of tropic acid, the direct precursor in both Scopolia and Datura cultures (Tabata et al., 1971). Zenk et al. (1975) reported that production of anthroquinones increased two-fold by the supply of a direct precursor O-succinylbenzoic acid. Changed conc of quaternary alkaloids were recorded in all the five strains of Ruta graveolens grown on media supplemented with tryptophan and precursors methylanthranilic acid 2,4-Dihydroxyquinoline (Ramawat et al., 1985). With Evolvulus alsinoides suspension cultures, tryptophan treatment resulted in higher production of alkaloids. Timing of addition of precursors was particularly important. Addition of tryptophan on days 5 and 10, enhanced appreciably the alkaloid production. On the other hand, later addition of tryptophan on day 15 and 20 had less stimulatory effect on alkaloid production (Nambiar, 1980).

However, the administration of a direct precursor is not necessarily effective in increasing the final yield e.g. in Lithospermum cultures, the addition of L-phenylalanine to the culture medium increased the shikonin content more than three fold, whereas the addition of more direct precursors including p-hydroxybenzoic acid failed to increase it (Tabata et al., 1976).

Incorporation problems with higher plants result from failure to transport precursor in their appropriate form to

the site of biosynthesis. The first serious biosynthetic studies with plant tissue cultures by using precursors were those of Ourisson, Benveniste and their colleagues. Their major contribution was to establish cycloartenol as the key intermediate to phytosterols, corresponding to lanosterol in animal steroid biosynthesis (Benveniste et al., 1966). Biosynthesis of mevalonoid derived compounds in cell cultures has been discussed in greater details by Overton (1977).

The understanding of the biosynthetic pathways of metabolites of commercial interest has been of great benefit in obtaining high concentrations of these metabolites e.g. since the early biosynthetic precursor of diosgenin are common to many other metabolites (phytol, carotenoids, chlorophyll, terpenes etc.) inhibition of the formation of one or more of these metabolites increased the level of diosgenin (Tal et al., 1984). Kaul et al. (1969) reported that the appropriate addition of cholesterol to suspension cultures of D. deltoidea raised the diosgenin production. Chowdhury and Chaturvedi (1979) reported that initial feeding of 100 mg l^{-1} cholesterol to Dioscorea deltoidea cultures increased diosgenin yields by 100%. Earlier Khanna and Manot (1976) reported that unorganized suspension culture of S. xanthocarpum when grown on RTO medium with various conc. of cholesterol, showed many fold increase in solasodine content.

Bhatt and Bhatt (1984 b) extensively studied the accumulation and fate of exogenous cholesterol as well as its effect on the rate of incorporation of acetate and mevalonate. Their results clearly showed that cholesterol significantly inhibits sterol synthesis in cell cultures of S. dalcamara from ^{14}C-acetate, but not from ^{3}H-mevalonate. Thus cholesterol controls sterol synthesis by the rate of production of mevalonate from acetate without affecting synthesis of other types of lipid from their precursors. The cholesterol feed back inhibition was also observed in the present studies (Chapter V-2.h).

Attempts were made to elucidate the effect of two stage culture medium on the sterol production. The separate factors exhibiting higher sterol content were combined together (production medium) to see whether they show any synergistic effect. Maximum % of sterol content was obtained when the cells were grown for 2 weeks in growth medium and then grown for 2 weeks in production medium. However, the sterol yield was maximum when the cells were grown for 3 weeks in growth medium and then 1 week in production medium because of the maximum biomass (Chapter V-2.i).

The growth medium and production medium are not necessarily the same. Sometimes they differ vastly. One of the most satisfactory methods currently available for achieving high accumulation of secondary metabolites per unit

of biomass is to employ a two-stage culture strategy. A first stage designed to produce high levels of biomass as rapidly as possible, followed by a second stage that is designed to stimulate metabolite production.

The rationale, therefore, would be to develop optimum growth conditions to obtain maximum cell mass accumulation followed by the induction of secondary product formation. Fujita et al. (1982) have developed a modified Lismaier-Skoog medium for optimal cell mass accumulation of Lithospermum erythrorhizon in large scale suspension cultures. The cells were shifted to a production medium that contained a 30-fold higher $CuSO_4$ conc; and as a result, in the production medium, the cells were able to synthesize much higher shikonin conc. It appears that, as with biotic stress conditions, the high $CuSO_4$ conc caused an apparent abiotic stress condition and induced the formation of secondary metabolite (Heinstein, 1985).

In the case of Dioscorea deltoidea cell cultures, it was found that the phosphate conc during the growth phase had a large influence on the later production of diosgenin; whereas if the phosphate was added in the production phase, there was no increase in diosgenin conc. Addition of sucrose directly to the second stage did not have any effect on biomass or diosgenin level, indicating that no external carbon source was required for biosynthesis of diosgenin in the production

phase (Rokem et al., 1985). This is similar to the result obtained by Heble and Staba (1980) who found that diosgenin was produced mainly by non-dividing cells.

Studies conducted with the passages of suspension culture revealed that even after 30th subculture, the cells exhibit the capacity to produce β-C-3 sterols with slight fluctuations at the 15th and 20th subculture. Slight decline in growth was observed after 20th subculture (Chapter V-2.j).

With regard to their production stability, there are at least two kinds of cell cultures. One kind produces the specific compounds in high yields with most or all the cells are producing. The cultures are high yielding from the beginning and are stable for many years e.g. Morinda citrifolia (Zenk et al., 1975). Second kind of cultures are clonally selected.

The conc of the active principle (guggulsterone-Z and E) was found to be less in the callus and suspension cultures, 0.009%, (Chapter V-3.a). Since β-C-3 sterols act as precursors for guggulsterones, a production medium was standardized for β-C-3 sterols which increased the β-C-3 sterol production. However, the high β-C-3 sterol pool did not result in higher pool of guggulsterones so another approach was tried.

There are reports of enhancement of gumresinosis by

ethephon (2-chloro ethyl phosphonic acid) in a number of woody plants (Bhatt and Shah, 1985). Gedalovich and Fahn (1985) observed prominent duct formation following the application of etherel in Citrus.

They have further reported that the pattern of differentiation and the ultrastructure of gum ducts formed after treatment with etherel was similar to that observed previously in stems infected with Phytophthora citrophthora. Stem segments artificially infected with P. citrophthora release ethylene and the amount released was highest about two days after infection. It was concluded that the production of ethylene by the infected stem tissue is a direct factor influencing duct development. There are other reports regarding the different actions of etherel which releases ethylene. It induced the formation of kino veins in Eucalyptus (Hillis, 1975), gum ducts in different members of Rosaceae (Wilde and Edjorton, 1975) and in Prosopis glandulosa (Greenwood and Morey, 1979).

This gave us a clue of trying the effect of ethephon in callus and suspension cultures. The guggulsterone content in both the suspension and callus cultures exhibited about 12.0 fold increase over the control after ethephon treatment (active principle - 2-chloroethylphosphonic acid, 100 mg l^{-1}) (Chapter V-3.b and V-3.c).

Ethylene is a natural plant growth regulator involved in the control of a wide range of developmental responses, growth abscission, senescence, fruit ripening and many others. However, the basic mechanism of action of the growth regulator at the molecular levels is still not clear. Sanders et al. (1986) proposed a hypothesis in which, the oxygenase would produce ethylene oxide which in turn would interact with the binding site (receptor) along with ethylene itself to bring about the appropriate biological response.

A large number of secondary natural products are phytoalexins, i.e. secondary metabolites synthesized in plants upon physical, chemical, microbiological or fungal damage of the plant. The signals that specify synthesis of secondary products in plant cells are elicitors and are associated with the invading microorganism and can induce the synthesis of the enzymes that catalyze the reactions in the pathway that lead to the secondary metabolite (Hahlbrock et al., 1981). e.g. addition of heat-denatured conidia of Verticillium dahliae to Gossypium arboreum cell cultures, caused a hundred-fold increase in conc of gossypol and its derivatives (Heinstein, 1982). This would indicate that by addition on nonviable V. dahliae conidia, a stress condition was initiated, causing induction of secondary product formation (Heinstein, 1985). Funk et al. (1987) isolated a carbohydrate fraction from yeast extract by ethanolic

preparation and it was used as an elicitor to induce glyceollin isomer synthesis in cells of Glycine max and enhancing berberine biosynthesis in cells of Thalictrum rugosum. Becker and Chavadej (1985) reported that colchicine treated suspension cultures of Valeriana wallichii produced higher amounts of Valeprotriates than did the respective untreated cultures. The ability to produce valeprotriates in the treated culture remained in the absence of colchicine even if the chromosome status returned to normal. Treatment of Ludino clover (Trifolium repens) callus with 1 mM p-chloromercuribenzoic acid (PCMBA) stimulated the biosynthesis of the phytoalexin medicarpin. The results also indicated that stimulation of medicarpin biosynthesis is reversible, but once activated, the pathway is not shut down by removal of elicitor (Gustine, 1987). Kurosaki et al.(1986) reported that defence responses of the whole plant including phytoalexin production and accumulation of phenotic acid were induced in cultured carrot cells. 6-methoxyellein, a phytoalexin of carrot, accumulated in cells treated with pectinase.

The totipotency of plant cell demonstrates that all the necessary genetic and physiological potential for product formation is present in the isolated cells. It therefore, appears reasonable to assume that during the transfers of plant cell cultures, the gene(s) which code(s) for a

specific enzyme or a number of enzymes in the pathway leading to the designed secondary product, became dormant or repressed. To correct this situation i.e. to de-repress the synthesis of the product, means have to be found somehow to induce the synthesis of secondary product. It appears, therefore, that stress conditions in plant cell cultures, can induce the formation of secondary products in cases where the cell cultures do not produce the desired compounds.

Restoration of the original secondary metabolite production during regeneration of plants from cell cultures have been observed in many cases.

But this type of production is not very efficient and requires greater length of time than that required for compound production by suspension cultures. It would be ideal if chemical differentiation is induced in cell cultures without morphological differentiation by artificial means.

An example of this is shikonin derivatives production. These derivatives are localized only in cork layers of the plant root, but cell cultures lacking cork cells also accumulate them (Tabata et al., 1974). According to Bychenkova (1973), resin which normally accumulates in the resin duct, was formed in tissue cultures of Pinus sylvestris. Although, resin formation decreased during successive subcultures, it remained constant at 1% of culture dry wt., after 13th passage.

Our results also indicate that the formation of guggulsterones, the major components of resin could be enhanced by the stress condition caused by ethephon. Whether the increase in guggulsterone production is due to a selection of high producing cells by ethephon, a gene amplification or another mechanism necessiates further investigations.

These findings are encouraging for future studies, and the manipulation of plant cells, plant genes and plant enzymes, rather than the plants themselves, should in future, provide us with a host of useful secondary metabolites.

* * * * *