

CHAPTER V - SECONDARY METABOLITES IN THE CULTURES

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## RESULTS

### CHAPTER V

#### SECONDARY METABOLITES IN THE CULTURES

- Experiments described in this chapter were performed with
- V-1 different parts of a plant and callus derived from these parts.
- V-2 suspension culture initiated from stem explants of Commiphora wightii was subjected to manipulation of cultural parameters for increased production of  $\beta$ -C-3 sterols. This was done with a view to raise the content of active principle Guggulsterones Z and E.
- V-3 Experiments were also conducted with Ethephon (2-chloro-ethylphosphonic acid) to trigger the synthesis of active principle in suspension and callus cultures.

V-1 TOTAL  $\beta$ -C-3 STEROL CONTENTV-1.a.1 Comparison of total sterol content of mature leaves, stem and callus derived from these explants

Commiphora wightii plants were grown in the University Botanical Garden from stem cuttings. Mature leaf and stem samples were collected from these plants. Leaves of the branch next to the youngest branch were fully expanded and were considered mature leaves. Stem samples of the same branch were collected. Fresh and dry weights of these explants were determined. Fresh and dry weights of callus derived from these explants were also determined. The samples were analyzed for total  $\beta$ -C-3 sterols as described in Materials and Methods (Chapter II-6.b).

Distribution of total sterols in Commiphora wightii indicates that on fresh weight basis the amount of total sterols was more in the stem explants than in mature leaves; so also the % of total sterols on dry weight basis (Table V-1).

Total sterols content of callus was significantly different from each other as well as the original explants used to initiate cultures. Callus cultures had a content of sterols about ten times lower than the original explants on fresh weight basis. However, on dry weight basis, the sterols content was only two to three times lower than the original explants.

Table : V-1

Comparison of Total Sterol Content of mature leaves,  
stem and callus tissues derived from these explants

Incubation : 36 days at  $25 \pm 2^\circ\text{C}$  (16 h, 1500 lux)

Medium : MS basal medium with 3% sucrose

Supplements : Kn -  $0.1 \text{ mg l}^{-1}$ , 2,4-D -  $0.1 \text{ mg l}^{-1}$   
Inositol  $100 \text{ mg l}^{-1}$

| Tissue           | Fresh wt.<br>(g) | Dry wt.<br>(g)    | Total Sterol<br>$\mu\text{g}/10 \text{ g fresh}$<br>wt. | % Dry wt. |
|------------------|------------------|-------------------|---|-----------|
| Mature<br>leaves | $10.1 \pm 0.46$  | $1.87 \pm 0.16$   | $4764 \pm 206.7$  | 0.256     |
| Callus           | $10.2 \pm 0.32$  | $0.405 \pm 0.017$ | $446 \pm 44.2$  | 0.110     |
| Stem             | $10.2 \pm 0.38$  | $2.12 \pm 0.18$   | $6232 \pm 268.1$  | 0.294     |
| Callus           | $10.1 \pm 0.36$  | $0.411 \pm 0.019$ | $568 \pm 58.1$  | 0.138     |

The stem derived callus exhibited 1.27 fold increase in the total sterol content than the leaf derived callus on fresh weight basis. The stem derived callus manifested 0.138% of total sterols on dry weight basis which is 1.25 fold more than the leaf derived callus which showed 0.110% of total sterols.

V-1.a.2 GC analysis of phytosterols of stem explants and callus derived from it

For Gas Chromatographic (GC) analysis, the digitonides were dissolved in pyridine and the regenerated sterols were redissolved in ethyl acetate. The GC analysis of this fraction was carried out after trimethylsilylating the sterols with BSA as mentioned in Chapter II-6.b. The fraction was resolved into three peaks. The sterols were identified by comparing their retention times with authentic standards (Fig. V-1). Thus, preliminary studies with GC analysis showed that the major components common in both the stem explants and callus initiated from it are cholesterol,  $\beta$ -sitosterol and campesterol.

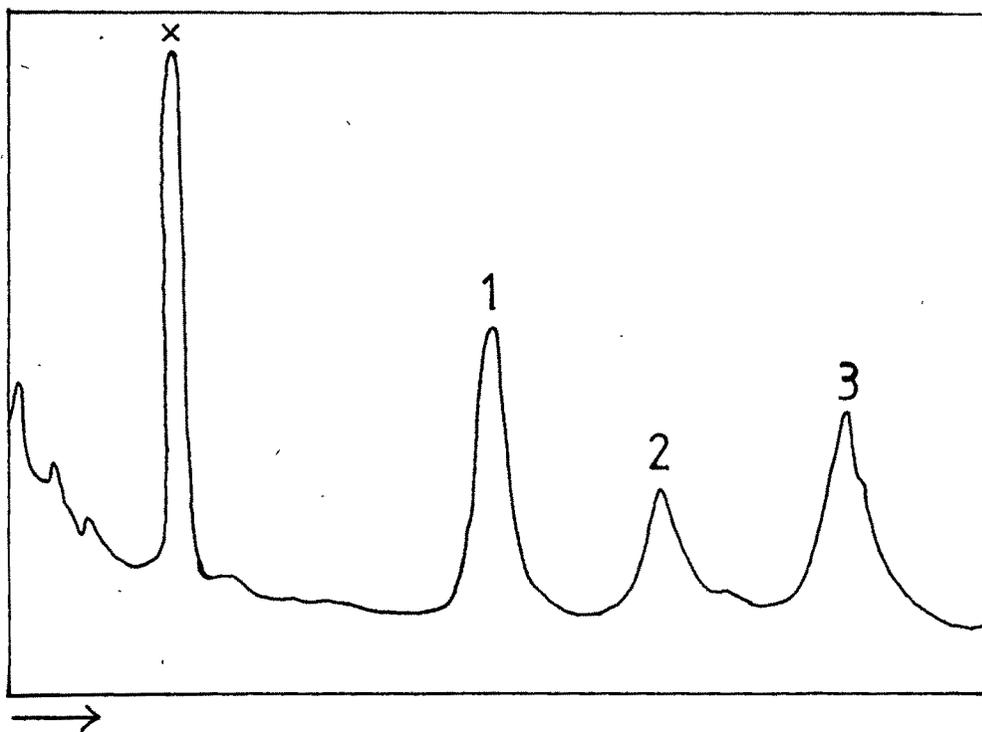


Fig. V-1 GC separation of  $\beta$ -C-3 sterols in callus culture of Commiphora wightii.

Steroidal constituents with retention times relative to  $5\alpha$ -cholastane (X) given in the parentheses are

- 1 : Cholesterol (2.06),
- 2 : campesterol (2.64),
- 3 :  $\beta$ -sitosterol (3.24).

As the total sterol content was more in the stem derived callus, subsequent experiments regarding the effect of various cultural parameters on the total  $\beta$ -C-3 sterol content were carried out using stem derived callus.

V-1. b Periodic changes in growth and total sterol levels in the standard growth medium in suspension cultures of *Commiphora wightii*

The standard growth medium in suspension culture denotes MS basal medium supplemented with Kn  $0.1 \text{ mg l}^{-1}$ , 2,4-D  $0.1 \text{ mg l}^{-1}$ ,  $100.0 \text{ mg l}^{-1}$  Inositol and 2% sucrose. Five ml ( $400 \pm 30 \text{ mg}$ ) cell suspension in stationary phase was transferred into 30 ml of medium. A fixed number of three replicates was harvested after four days intervals till day 24 for determining growth of tissue and sterol production. The results are presented in the Fig. V-2.

The growth of cells in suspension culture exhibited an initial lag phase of 8 days, followed by a rapid period of growth till day 16. The growth was almost stationary till day 20, thereafter it declined. During the exponential phase, the cells in suspension culture manifested 25.7 fold increase in fresh weight and 25.0 fold increase in dry weight. The kinetics of total sterol content suggests that on per cent wise, the total sterol content drops during lag phase, increases slowly during exponential and stationary phases and

thereafter it almost remains constant. Culture-wise, a slow increase during initial 8 days is followed by a rapid increase in sterol content till day 20, after which it declines slightly.

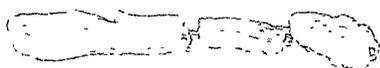
## V-2 INFLUENCE OF VARIOUS CULTURAL PARAMETERS ON GROWTH AND TOTAL STEROL CONTENT

Prior to the transfer of suspension culture on experimental media, the cells were grown in MS basal medium for one week, unless otherwise mentioned, in order to minimize hormonal carryover. Size of the inoculum on experimental medium was 5 ml cell suspension ( $400 \pm 30$  mg by fresh wt. and  $12.5 \pm 2$  mg by dry wt.). The cultures were kept on a gyratory shaker at 125 rpm at  $25 \pm 2^\circ\text{C}$  and at 16 h photoperiod, 1200 lux. The cultures were incubated for 24 days. At the end of the culture period, they were analysed for fresh and dry wt. increases and also total sterol accumulation. The results are presented in Tables V-2-V-4 and Fig. (V-3-V-8).

### V-2.a Effect of total nitrate level on growth and total sterol content in suspension culture

In the present studies, the influence of various levels of nitrate in the form of  $\text{NH}_4\text{NO}_3$  and  $\text{KNO}_3$  was investigated. MS basal medium contained 40 mM of nitrate ( $20.6$  mM  $\text{NH}_4\text{NO}_3$  and  $18.8$  mM  $\text{KNO}_3$ ). This nitrate level has been altered to investigate its effect on growth and total sterol content of cells. For the sake of convenience, the level of  $\text{NH}_4\text{NO}_3$  and

$\text{KNO}_3$  is arbitrarily taken as 20.0 mM. When the level of  $\text{KNO}_3$  was reduced to one half i.e. 10 mM, equivalent amount of K was added in the form of KCl. Citrate at 10 mM was added in the media with X1.5 and X2.0 level of nitrate to absorb the excess of ammonium. The results are presented in the Fig.V-3.



The % total sterol content enhanced with increasing level of nitrate and was 1.98 fold more than the control at 80 mM nitrate (40 mM of  $\text{NH}_4\text{NO}_3$  and 40 mM of  $\text{KNO}_3$ ). The increased nitrate level, however, drastically reduced the cell growth, 0.06 fold less on fresh weight basis and 0.058 fold less on dry weight basis than in control. The X0.5 level of nitrate ( $\text{NH}_4\text{NO}_3$  10 mM and  $\text{KNO}_3$  10.0 mM) supported growth of cells and it showed 1.06 fold increase on fresh weight basis and 1.08 fold increase on dry weight basis. However, the total sterol content dropped from 0.134% to 0.125%.

These results clearly showed that the increased levels of nitrate supported sterol production but drastically inhibited cell growth.

Fig. V-2 Time course of growth and total sterol production in MS + Kn ( $0.1 \text{ mg l}^{-1}$ ) + 2,4-D ( $0.1 \text{ mg l}^{-1}$ ) + Inositol ( $100 \text{ mg l}^{-1}$ ).  
Incubation : 24 days at  $25 \pm 2^\circ \text{C}$  (16 h, 1000 lux).

Fig. V-3 Effect of Nitrate level on growth and total sterol content in suspension culture.  
Medium : MS (without nitrate) + Kn ( $0.1 \text{ mg l}^{-1}$ ) + 2,4-D ( $0.1 \text{ mg l}^{-1}$ ) + Inositol ( $100 \text{ mg l}^{-1}$ ).  
Nitrate added at different levels.  
Incubation : 24 days at  $25 \pm 2^\circ \text{C}$  (16 h, 1000 lux).

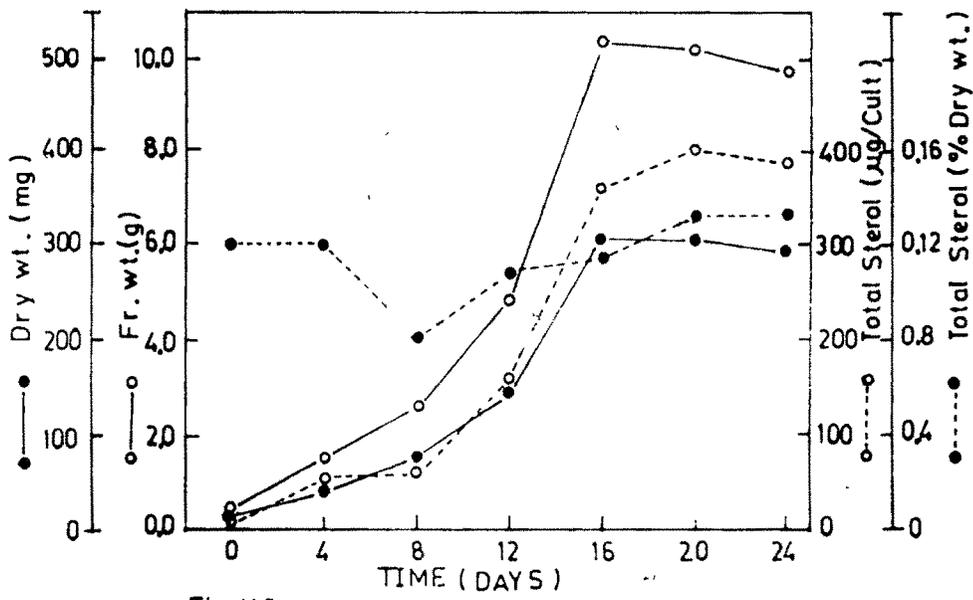


Fig. V-2

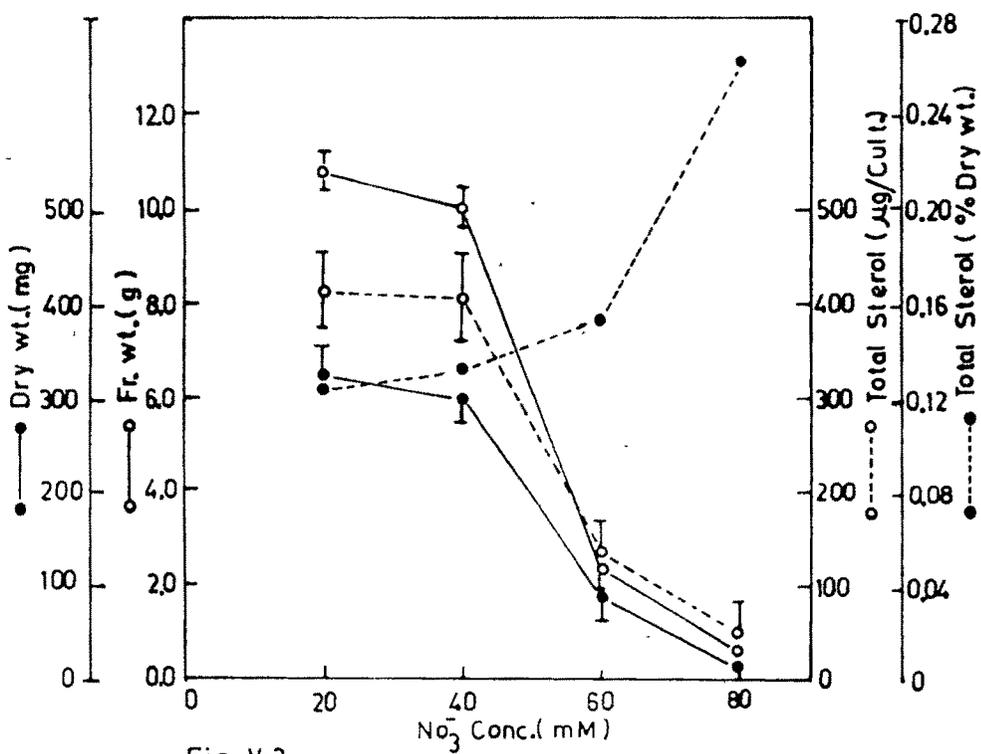
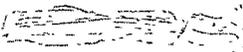


Fig. V-3

V-2, b Effect of phosphate level on growth and total sterol content in suspension culture

MS basal medium contains inorganic phosphate in the form of Potassium dihydrogen Orthophosphate ( $\text{KH}_2\text{PO}_4$ ) at  $170 \text{ mg l}^{-1}$ . Its level was altered.  $\text{KH}_2\text{PO}_4$  was incorporated in the medium at  $85.0 \text{ mg l}^{-1}$  (X0.5),  $255 \text{ mg l}^{-1}$  (X1.5) and  $340 \text{ mg l}^{-1}$  (X2.0) level.

The results presented in  Fig. V-4 showed that reduction in the phosphate level, depressed the growth of the cells. Moreover, it also depressed the total sterol content. Fold wise decrease in growth on fresh weight basis was 0.61 and on dry weight basis was 0.822. Total sterol content (%) was only 0.649 fold as compared with the control. As more phosphate was incorporated in the medium, there was increase in growth (both on fresh and dry weight basis) and also total sterol content. At X1.5 phosphate level, fold wise increase in fresh and dry weight was 1.06. At the same phosphate level, the maximum sterol content was registered, 0.213% wise and  $678.1 \mu\text{g}$  culture wise, showing 1.6 fold increase as compared with the control.

Further increase in phosphate level, X2.0,  $340 \text{ mg l}^{-1}$ , however, reduced the growth of cells. It also reduced the total sterol content and it was only 1.2 fold more than the control.

V-2.c Effect of different carbon sources on growth and sterol content in suspension culture

Carbohydrates, not only provide the energy source, but also supply carbon skeleton for the synthesis of secondary metabolites in plants. Experiments were designed, therefore, to find out the suitable carbohydrate as energy source and carbon source for sterols and its optimal concentration.

The standard growth medium which lacked sucrose, was supplemented with the monosaccharides glucose, fructose, galactose, xylose, disaccharides sucrose, maltose, lactose and polysaccharide starch. The cells were grown in standard medium lacking sucrose for 4 days to minimise the carryover effect. The results are summarized in Table V-2.

Some carbon sources fructose, glucose supported growth but not product formation. However, the growth was less than sucrose. Some carbon sources such as starch, galactose and lactose helped sterol production but in lower amounts than those obtained with sucrose. The other carbon sources such as maltose and xylose did not help product formation. Moreover, the growth was also poor with these carbohydrates.

Thus sucrose was found to be the most suitable carbon source for growth and sterol production.

V-2.d Effect of sucrose levels on growth and sterol production in suspension cultures

To determine the optimal sucrose level for sterol production, the standard growth medium was supplemented with various sucrose levels ranging from 1 to 8%.

As indicated in  Fig. V-5 the cells did not grow at all in the medium containing no sucrose. Addition of 1.0% sucrose enhanced the growth of cells as well as sterol production considerably and the growth (both on fresh and dry weight basis) was maximum at 2.0% sucrose. Further increase in the sucrose level reduced the growth of cells. However, the total sterol content steadily increased with increasing concentrations of sucrose and culture wise it was maximum at 4.0% sucrose (624  $\mu\text{g}/\text{cult.}$ ), whereas on % wise it was maximum (0.301%) at 6.0% sucrose. The sterol content dropped with further increase in sucrose level.

V-2.e Effect of auxins on growth and sterol content in suspension cultures

The nature of hormones as well as their concentrations influence the ability of the cells to produce metabolites. The different auxins studied for their effect on growth and sterol production were 2,4-D, NAA, IAA and IBA. Each auxin was used at the concentration, 0.01, 0.1 and 1.0  $\text{mg l}^{-1}$  in standard medium without 2,4-D.

Fig. V-4 Effect of phosphate level on growth and total sterol content in suspension culture.

Medium : MS (without phosphate) + Kn ( $0.1 \text{ mg l}^{-1}$ )  
+ 2,4-D ( $0.1 \text{ mg l}^{-1}$ ) + Inositol  
( $100 \text{ mg l}^{-1}$ ).

Phosphate added at different levels.

0.625 mM (X 0.5)

1.25 mM (X 1.0)

1.875 mM (X 1.5)

2.5 mM (X 2)

Incubation : 24 days at  $25 \pm 2^\circ\text{C}$  (16 h, 1000 lux).

Fig. V-5 Effect of sucrose conc. on growth and total sterol content in suspension culture.

Medium : MS (without sucrose) + Kn ( $0.1 \text{ mg l}^{-1}$ )  
+ 2,4-D ( $0.1 \text{ mg l}^{-1}$ ) + Inositol  
( $100 \text{ mg l}^{-1}$ ).

Sucrose added at different concs.

Incubation : 24 days at  $25^\circ\text{C}$  (16 h, 1000 lux).

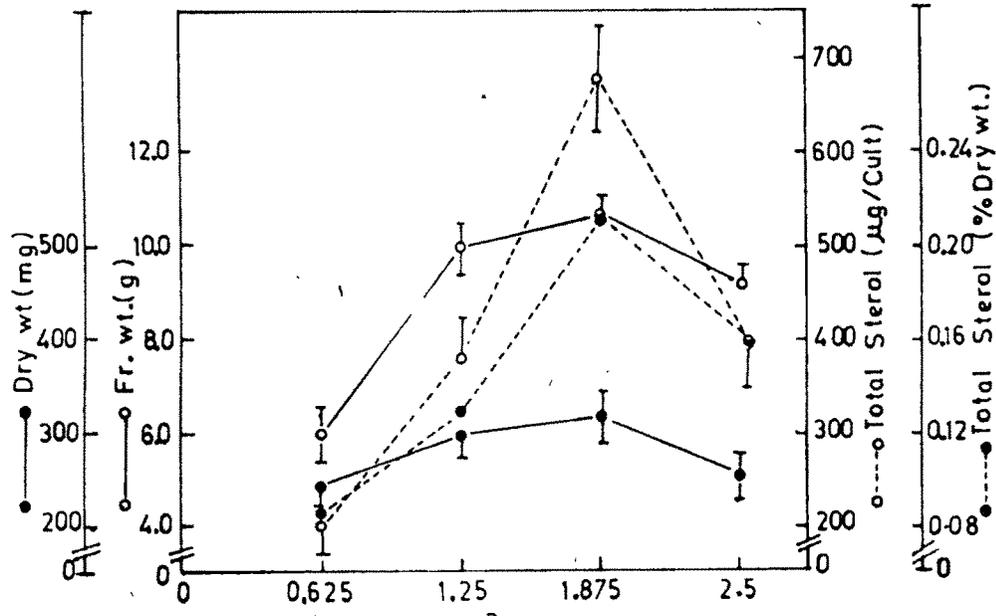


Fig. V-4 INITIAL  $PO_4^{3-}$  Conc. (mM)

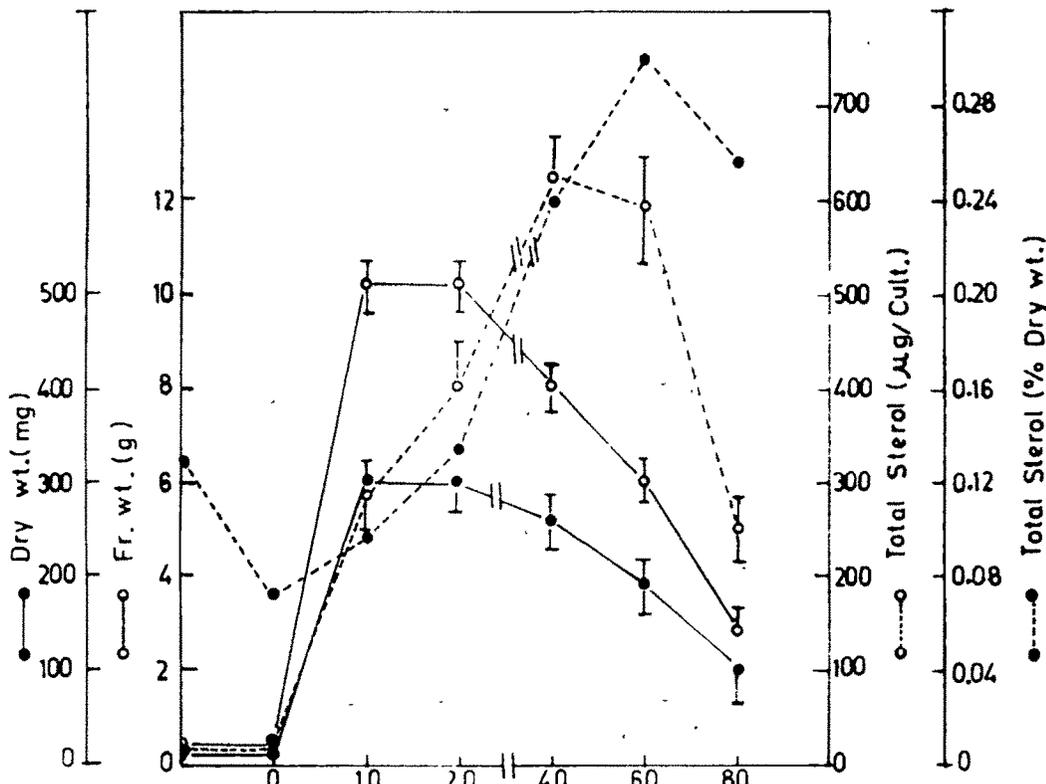


Fig. V-5

Sucrose Conc (%)

Table : V-2

Effect of different carbon sources on growth and sterol content in suspension culture.

Inoculum : 400±30 mg tissue by fresh wt.

Medium : MS basal medium without sucrose

Supplements: Kn - 0.1 mg l<sup>-1</sup>, 2,4-D - 0.1 mg l<sup>-1</sup>, Inositol - 100 mg l<sup>-1</sup>

All carbon sources added at 2% concentration.

Incubation : 24 days at 25±2°C (16 h, 1000 lux)

| Carbon source | Fresh wt.<br>mg/cult. | Dry wt.<br>mg/cult. | Total Sterol Content |              |
|---------------|-----------------------|---------------------|----------------------|--------------|
|               |                       |                     | μg/cult.             | % of dry wt. |
| 1. Sucrose    | 10226.8(±196.8)       | 306.8(±14.8)        | 413.1(±36.8)         | 0.135        |
| 2. Fructose   | 9664.3(±176.6)        | 288.4(±14.11)       | 100.8 (±20.1)        | 0.035        |
| 3. Glucose    | 9886.5(±182.4)        | 292.5(±13.9)        | 110.1(±20.4)         | 0.038        |
| 4. Maltose    | 862.4(± 46.2)         | 28.8(± 9.6)         | 12.6(± 6.8)          | 0.045        |
| 5. Lactose    | 504.3(±25.4)          | 18.1(±2.21)         | 13.5(±5.4)           | 0.075        |
| 6. Galactose  | 502.8(±24.8)          | 17.8(±2.4)          | 12.1(±5.6)           | 0.068        |
| 7. Xylose     | 615.3(±30.2)          | 19.2(±3.1)          | 8.06(±5.2)           | 0.042        |
| 8. Starch     | 501.4(±20.8)          | 16.9(±1.9)          | 13.6 (±6.4)          | 0.085        |

Data represents an average of 3 replicates.

Figures in the parenthesis represent standard deviation.

Results indicating the effect of auxins concentration are presented in ~~Fig. V-6~~ Fig. V-6 a,b,c,d. At  $0.1 \text{ mg l}^{-1}$  of 2,4-D, the total sterol content was 0.136% and  $416.2 \mu\text{g/culture}$  at the end of the incubation period. At the reduced level of 2,4-D ( $0.01 \text{ mg l}^{-1}$ ), the growth of the cells was slightly suppressed, however, the total sterol content increased, showing 0.230% and  $593.4 \mu\text{g/culture}$ . Thus showing 1.6 fold increase % wise and 1.4 fold increase culture wise. At higher concentration  $1.0 \text{ mg l}^{-1}$ , both growth and total sterol production were reduced.

In the case of IAA, it did not support the growth of the tissue and the highest growth obtained with IAA at  $0.01 \text{ mg l}^{-1}$  concentration ( $1.12 \text{ g/culture}$ ) is only 0.112 fold compared with the control. With increasing concentration of IAA  $1.0 \text{ mg l}^{-1}$  through  $0.1 \text{ mg l}^{-1}$ , the growth of cells declined both on fresh and dry wt. basis. However, the increasing concentration of IAA supported sterol production and at  $1.0 \text{ mg l}^{-1}$ , it was 0.185 % ( $51.8 \mu\text{g/culture}$ ), which is 1.36 fold more than control.

Of the three levels of IBA administered, the cells exhibited maximum growth  $2.1 \text{ g/cult.}$  at the lowest concentration  $0.01 \text{ mg/l}$ . The growth declined with increase in the concentration. However, the sterol content <sup>(%wise)</sup> increased with the increasing concentration and was 0.110% ( $52.8 \mu\text{g/cult}$ ) which is 0.80 fold as compared with control.

NAA supported growth of the cells (3.6 g/cult) and total sterol production (0.110%) more than the other two auxins, IAA and IBA at the lowest concentration ( $0.01 \text{ mg l}^{-1}$ ). However, the increasing concentrations of NAA exhibited decline in the growth of cells and also total sterol production (on culture basis).

These results presented in Fig. V-7 a,b,c,d indicate that 2,4-D at  $0.1 \text{ mg l}^{-1}$  is optimal for growth of the cells on fresh as well as dry wt. basis. However, the total sterol content fluctuated at the different concentrations. At the lowest concentration, 2,4-D promoted the highest sterol content 0.230% ( $593 \mu\text{g/cult.}$ ) followed by NAA 0.110% ( $123.2 \mu\text{g/cult.}$ ), IBA 0.105% ( $68.2 \mu\text{g/cult.}$ ) and IAA 0.095% ( $38.8 \mu\text{g/cult.}$ ). However, at the higher concentration, 1.0 mg/lit, the pattern was reversed, thus showing maximum sterol content with IAA followed by 2,4-D, NAA and IBA.

V-2.f Influence of auxin 2,4-D and cytokinin kinetin on growth and sterol production in suspension culture

To examine the effect of 2,4-D and kinetin in combination on growth and total sterol content the following combinations were studied :-

|       |      | Kn      |     |     |
|-------|------|---------|-----|-----|
|       |      | 0.01    | 0.1 | 1.0 |
| 2,4-D | 0.01 |         |     |     |
|       | 0.1  | Control |     |     |
|       | 1.0  |         |     |     |

Fig. V-6 Influence of auxins 2,4-D (6.a), IAA (6.b), IBA (6.c) and NAA (6.d) on growth and total sterol content in suspension culture.

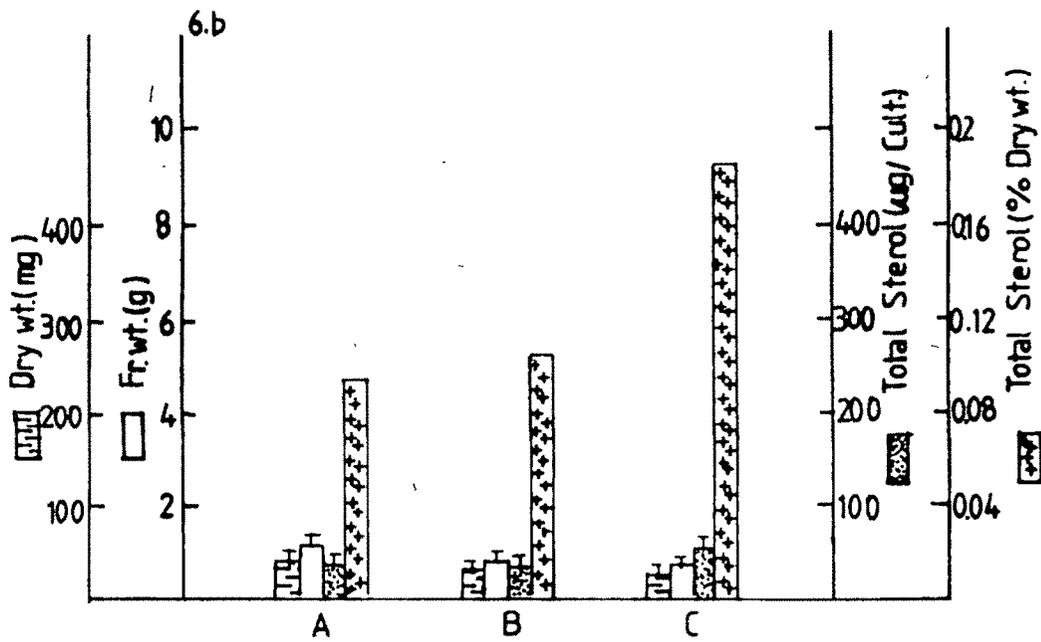
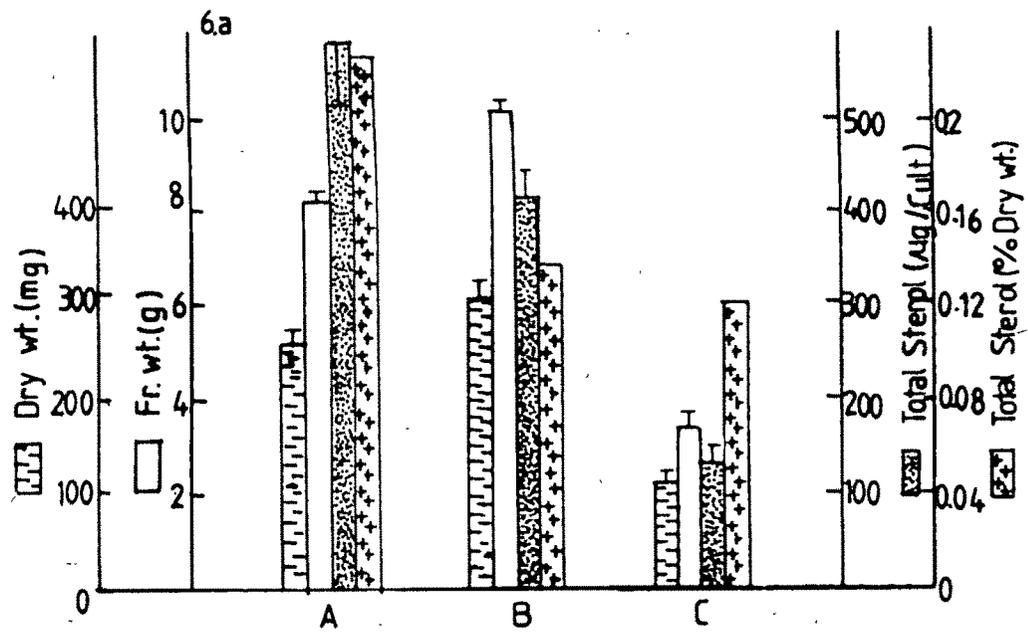
Medium : MS + Kn ( $0.1 \text{ mg l}^{-1}$ ) + inositol ( $100 \text{ mg l}^{-1}$ ).

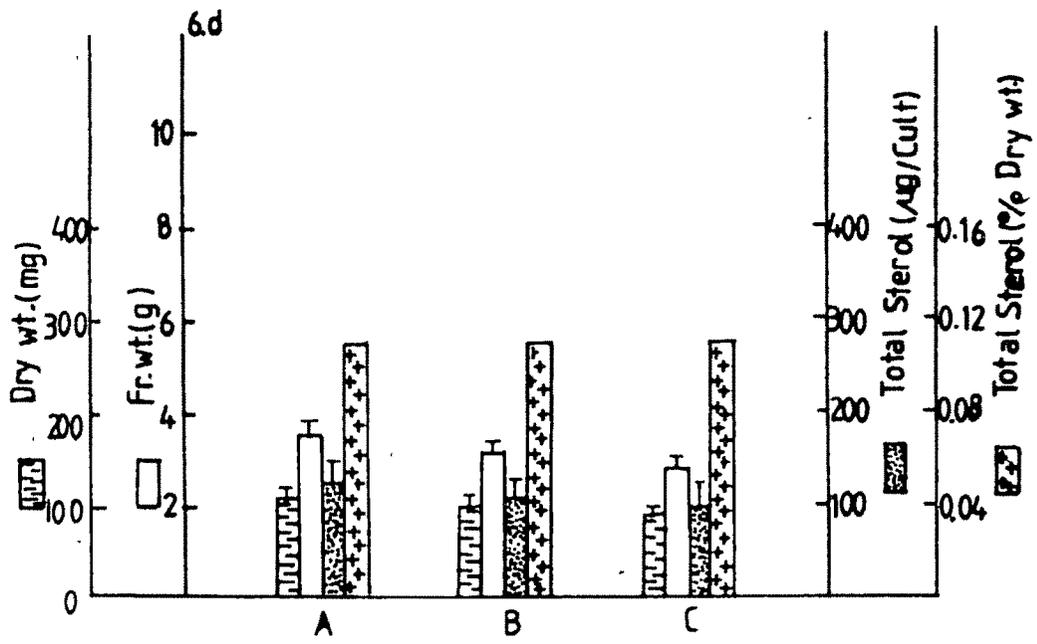
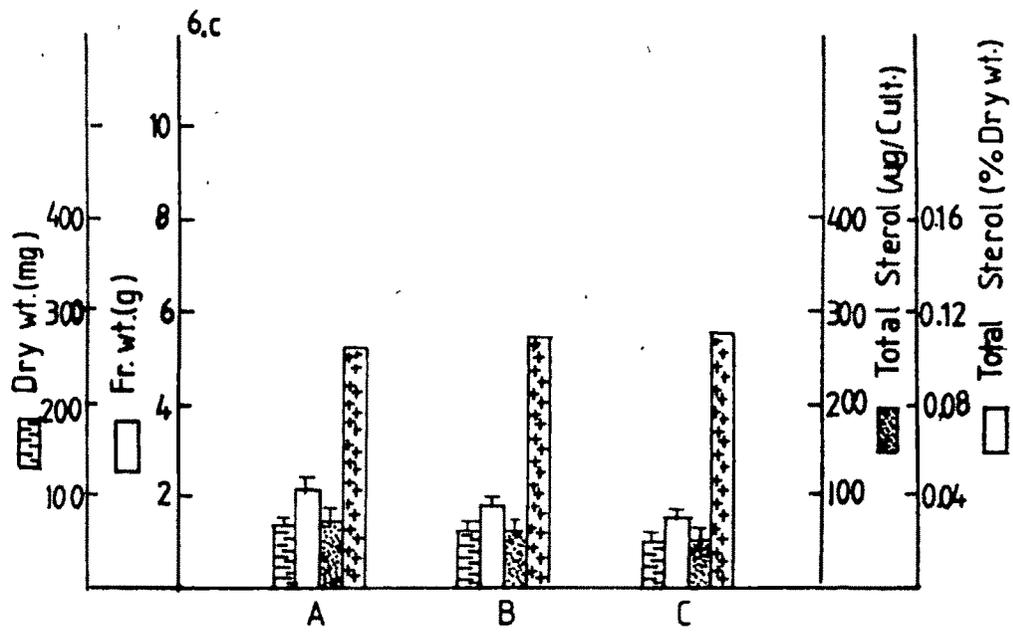
Auxins added at different concs.

A -  $0.01 \text{ mg l}^{-1}$ , B -  $0.1 \text{ mg l}^{-1}$ ,

C -  $1.0 \text{ mg l}^{-1}$ .

Incubation : 24 days at  $25^\circ\text{C}$  (16 h, 1000 lux).





The results obtained are presented in ~~Figure 7.2, 7.3, 7.4~~  
 Fig. V-7.a, b, c.

Of the various combinations of 2,4-D and kn tried, 2,4-D at  $0.1 \text{ mg l}^{-1}$  with kn at  $0.1 \text{ mg l}^{-1}$  gave the maximum cell yield both on fresh and dry wt. basis. It was also observed that the growth of the cells was more influenced by the concentration of 2,4-D than kn. However, the maximum sterol content was obtained at  $0.01 \text{ mg l}^{-1}$  2,4-D with  $0.1 \text{ mg l}^{-1}$  kn (0.236%, 535  $\mu\text{g/cult.}$ ) which is 1.7 fold more than the control on % wise and 1.3 fold more on  $\mu\text{g/cult.}$  basis. Irrespective of the kn concentration, the total sterol content (% dry wt.) was always higher at the lower concentration of 2,4-D ( $0.01 \text{ mg l}^{-1}$ ) than the control.

So, it is observed that the growth as well as total sterol content was more influenced by the concentration of auxin (2,4-D) than that of cytokinin (kn).

V-2.g Effect of different levels of Micro and Macro element salts on growth and sterol production

The combinations of macro element and micro element salts studied were :

|                     |       | Macro element salts |       |       |
|---------------------|-------|---------------------|-------|-------|
|                     |       | x 0.5               | x 1.0 | x 2.0 |
| Micro element salts | x 0.5 |                     |       |       |
|                     | x 1.0 | Control             |       |       |
|                     | x 2.0 |                     |       |       |

Fig. V-7 Effect of 2,4-D and Kn on growth and sterol production in suspension culture.

Medium : MS + inositol ( $100 \text{ mg l}^{-1}$ ).

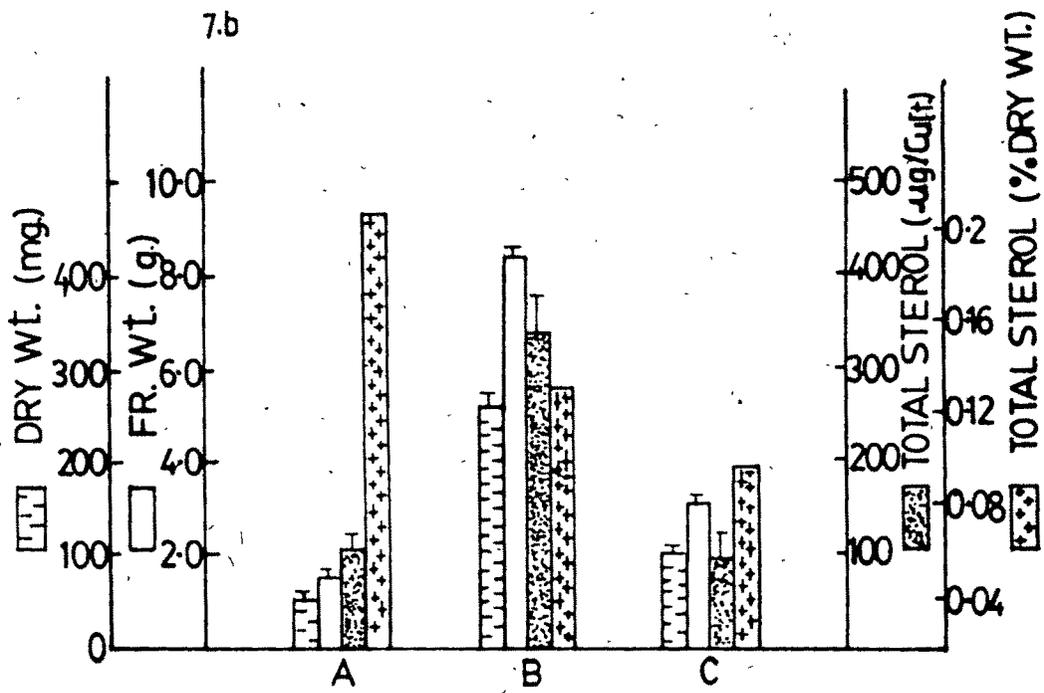
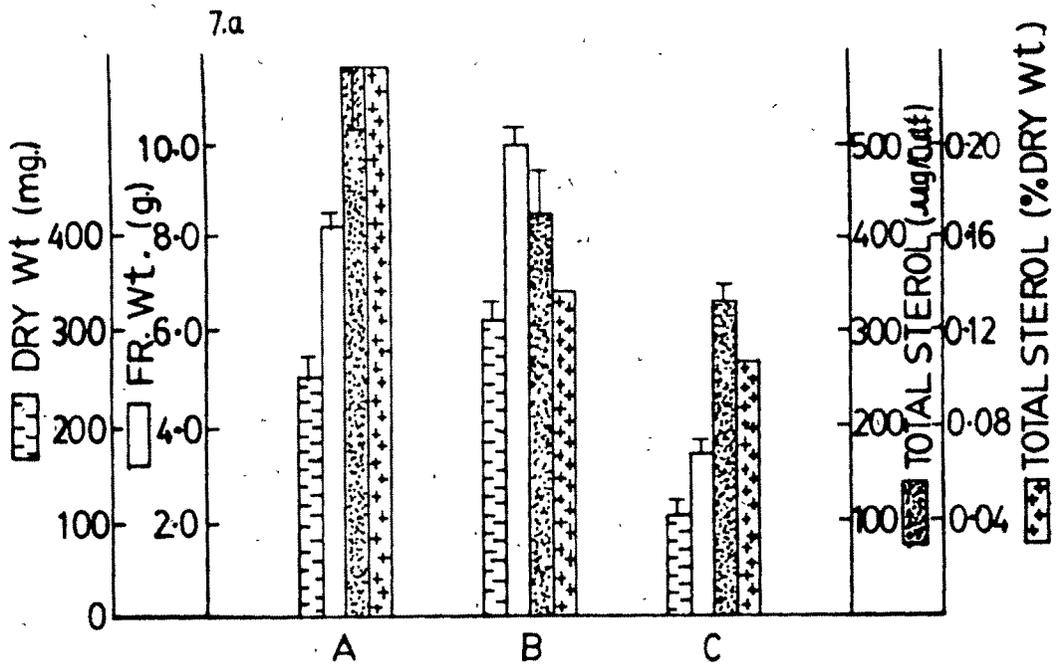
2,4-D and Kn added at different concs.

Kn concs. 7.a : -  $0.1 \text{ mg l}^{-1}$ , 7.b : -  $0.01 \text{ mg l}^{-1}$ , 7.c : -  $1.0 \text{ mg l}^{-1}$ .

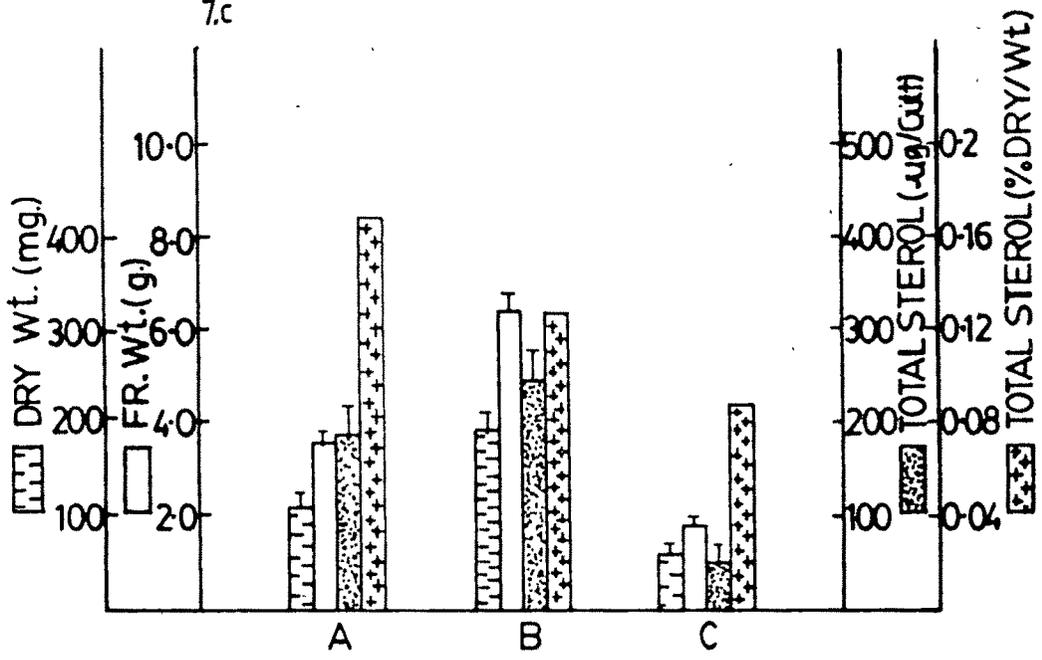
2,4-D concs.

A -  $0.01 \text{ mg l}^{-1}$ , B -  $0.1 \text{ mg l}^{-1}$ ,

C -  $1.0 \text{ mg l}^{-1}$ .



7.c



In macroelements, the level of nitrate ( $\text{NH}_4\text{NO}_3$  and  $\text{KNO}_3$ ) and phosphate ( $\text{KH}_2\text{PO}_4$ ) was kept constant and the level of other macroelements viz.  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  ( $440 \text{ mg l}^{-1}$ ),  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  ( $370 \text{ mg l}^{-1}$ ) and  $\text{Na}_2\text{FeEDTA}$  ( $\text{Na}_2\text{EDTA}$  37.3 and  $\text{FeSO}_3$  278  $\text{mg l}^{-1}$ ) was varied. In microelements the level of  $\text{MnSO}_4$  ( $22.3 \text{ mg l}^{-1}$ ),  $\text{ZnSO}_4$  ( $8.6 \text{ mg l}^{-1}$ ),  $\text{H}_3\text{BO}_3$  ( $6.2 \text{ mg l}^{-1}$ ),  $\text{KI}$  ( $0.83 \text{ mg l}^{-1}$ ),  $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$  ( $0.25 \text{ mg l}^{-1}$ ),  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  ( $0.025 \text{ mg l}^{-1}$ ) and  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$  ( $0.025 \text{ mg l}^{-1}$ ) was varied.

As indicated in Table V-3 ~~at the x0.5 level~~ at the x0.5 level of macro and micro elements, the growth of cells was suppressed so also the total sterol content. Incorporation of microelements at increasing levels at X0.5 level of macroelements did support growth and total sterol content, but it was still less than the control.

By increasing the level of macroelements to X1.0, both, the growth of cells and total sterol content was enhanced. Maximum growth of cells on fresh and dry wt. basis, was obtained at X1.0 of macro and microelements. Further increase in macroelements to X2.0 had adverse effect on cell growth; so also at all levels of microelements. However, the total sterol content was slightly enhanced at X2.0 macro + X1.0 micro and X2.0 of macro and X2.0 of microelements (0.137%) showing 1.03 fold increase over the control.

Table : V-3

Effect of different levels of micro and macroelement salts on growth and sterol production

Inoculum : 400 $\pm$ 30 mg tissue by fresh wt.

Medium : Vitamin stock of MS medium with 2% sucrose

Supplements: Kn - 0.1 mg l<sup>-1</sup>, 2,4-D - 0.1 mg l<sup>-1</sup>,

Inositol - 100 mg l<sup>-1</sup>

macro and micro salts added at different levels.

Incubation : 24 days at 25 $\pm$ 2°C (16 h, 1000 lux)

| Additives<br>(X) | Fresh wt.<br>mg/cult.     | Dry wt.<br>mg/cult.    | Total sterol content   |           |
|------------------|---------------------------|------------------------|------------------------|-----------|
|                  |                           |                        | $\mu$ g/cult.          | % dry wt. |
| Macro    Micro   |                           |                        |                        |           |
| 0.5 + 0.5        | 1108.2<br>( $\pm$ 62.1)   | 35.9<br>( $\pm$ 4.1)   | 11.5                   | 0.031     |
| 0.5 + 1.0        | 6587.4<br>( $\pm$ 178.1)  | 208.5<br>( $\pm$ 11.8) | 135.1<br>( $\pm$ 29.6) | 0.064     |
| 0.5 + 2.0        | 7149.1<br>( $\pm$ 192.6)  | 222.5<br>( $\pm$ 12.8) | 157.6<br>( $\pm$ 30.2) | 0.070     |
| 1.0 + 0.5        | 3536.4<br>( $\pm$ 108.4)  | 92.3<br>( $\pm$ 9.8)   | 75.5<br>( $\pm$ 10.3)  | 0.082     |
| 1.0 + 1.0        | 10215.7<br>( $\pm$ 288.2) | 318.2<br>( $\pm$ 20.2) | 424.3<br>( $\pm$ 46.2) | 0.133     |
| 1.0 + 2.0        | 9106.6<br>( $\pm$ 236.4)  | 312.4<br>( $\pm$ 19.8) | 426.2<br>( $\pm$ 41.7) | 0.136     |
| 2.0 + 0.5        | 3586.1<br>( $\pm$ 112.3)  | 86.8<br>( $\pm$ 9.6)   | 79.9<br>( $\pm$ 10.6)  | 0.093     |
| 2.0 + 1.0        | 4086.9<br>( $\pm$ 124.1)  | 126.2<br>( $\pm$ 12.8) | 172.6<br>( $\pm$ 32.4) | 0.137     |
| 2.0 + 2.0        | 4008.3<br>( $\pm$ 119.6)  | 122.3<br>( $\pm$ 13.2) | 167.1<br>( $\pm$ 28.8) | 0.137     |

The data represents an average of 3 replicates.

Figures in the parenthesis represent standard deviations.

V-2.h Effect of precursors on growth and sterol production  
in *Commiphora wightii* suspension cultures

The metabolic sequence of sterol biosynthesis in plants is similar to that of cholesterol biosynthesis in animal systems (Grunwald, 1975). The main precursors of sterols are acetate, mevalonate and squalene. The regulation of cholesterol biosynthesis is also complex. The rate limiting step is early in the sequence; the reaction in which hydroxymethyl glutaryl-CoA is converted into mevalonate. The enzyme catalyzing this reaction hydroxymethylglutaryl CoA reductase is a complex regulating enzyme. Cholesterol inhibits its own synthesis by regulating the activity of HMG CoA reductase.

Keeping this in view, experiment was set up to study the effect of sodium acetate, sodium mevalonate and cholesterol on the total sterol content of *Commiphora wightii* suspension cultures.

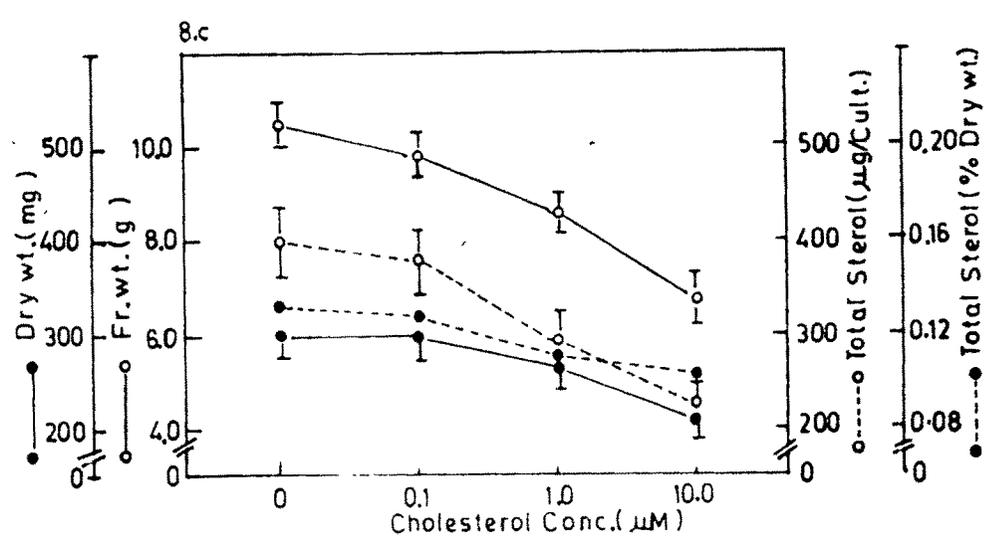
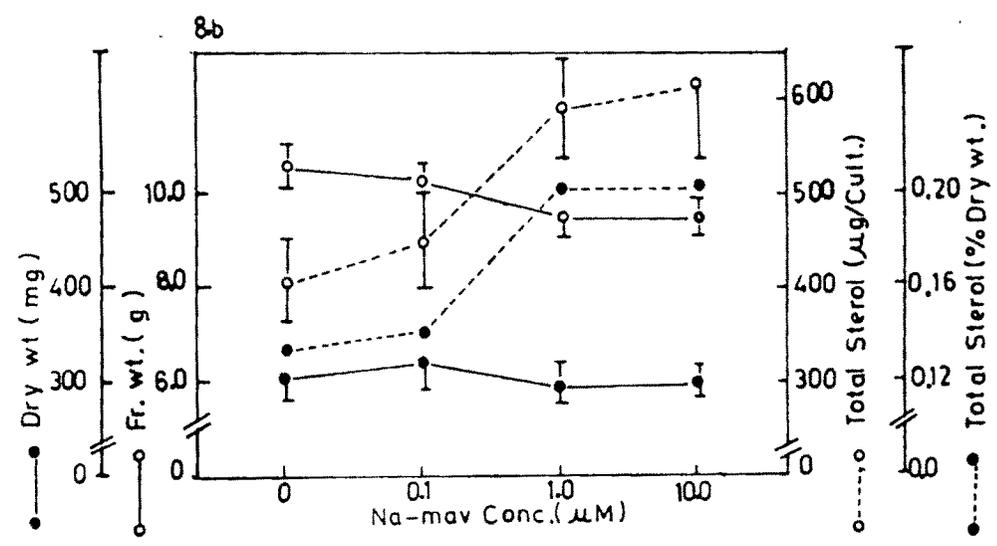
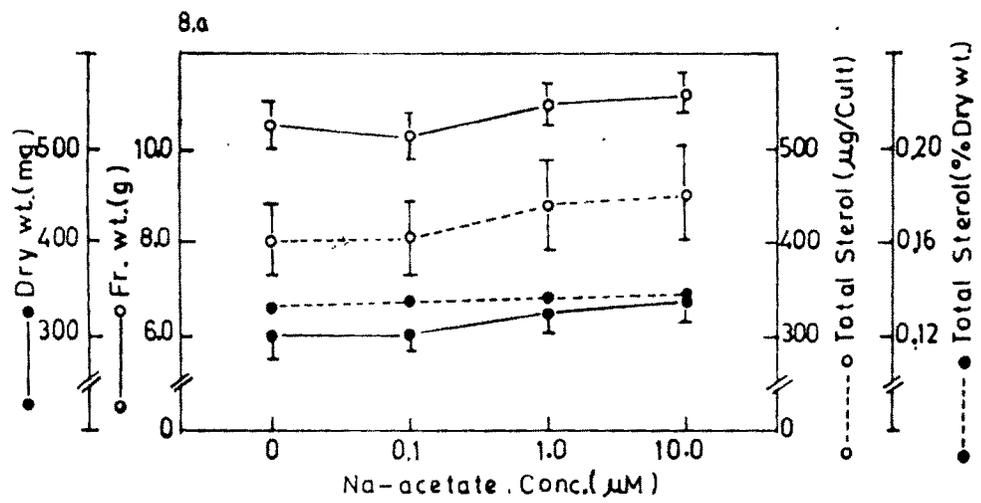
Effect of the precursors of the sterol biosynthesis by adding sodium acetate and sodium mevalonate, in the medium at 0.1, 1.0 and 10.0  $\mu$ M concentration was then examined. In another set of experiments, cholesterol was added in the medium at 0.1, 1.0 and 10.0  $\mu$ M concentrations for its effect on total sterol content (Fig.V-8.a,b,c).

Addition of sodium acetate had a promotory effect on growth as well as total sterol content. However, the total

Fig. V-8 Effect of precursors, Na-acetate (8.a),  
Na-mavlonate (8.b) and cholesterol (8.c)  
on growth and sterol content in suspension  
culture.

Medium : MS + Kn ( $0.1 \text{ mg l}^{-1}$ ) + 2,4-D ( $0.1 \text{ mg l}^{-1}$ )  
+ inositol ( $100 \text{ mg l}^{-1}$ ).

Incubation : 24 days at  $25^{\circ}\text{C}$  (16 h, 1000 lux).



sterol content was only 1.02 fold more than control at all the three levels of sodium acetate tested.

Incorporation of sodium mavlonate in the increasing levels markedly increased the total sterol level (0.2% at 1.0 and 10.0  $\mu\text{M}$  of sodium mavlonate) with a slight inhibition of growth of cells. With cholesterol, however, a drop in total sterol content was noticed which increased with the increasing concentration of cholesterol. The growth of the cells was also affected by cholesterol.

This seemed to indicate that the immediate precursor sodium mavlonate was utilized by the cell culture more effectively for the production of sterols than sodium acetate. Cholesterol inhibited the total sterol content.

V-2.1 Effect of two stage culture system on growth and sterol production in suspension cultures of *Commiphora wightii*

It has been observed that a two stage culture system is beneficial for the production of secondary metabolites. In the present studies, an experiment was designed to find out the optimum timing for the transfer of cells from growth medium to production medium. Here the growth medium denotes MS with kn  $0.1 \text{ mg l}^{-1}$ , 2,4-D  $0.1 \text{ mg l}^{-1}$ , inositol  $100 \text{ mg l}^{-1}$  and 2% sucrose; whereas in production medium, the separate

factors exhibiting higher sterol content were pooled together to see whether they showed any synergistic effect. The production medium denotes MS medium (without nitrate and phosphate source) with X2.0 nitrate, X1.5 phosphate, 2,4-D  $0.01 \text{ mg l}^{-1}$ , kn  $0.1 \text{ mg l}^{-1}$  and sucrose 6%.

The cells were grown first in the growth medium and successively transferred to production medium at different periods. The cells were harvested after 4 weeks. As presented in Table V-4 in control, i.e. when the cells were grown only in growth medium for all the four weeks, the growth of the cells was maximum and it registered total sterol content of 0.133%. When the cells were grown in the growth medium for one week and then in the production medium for 3 weeks, the growth of the cells was drastically reduced. It was 0.24 fold as compared with the control on fresh wt. basis and 0.205 fold on dry wt. basis. Even though the total sterol content was less than in control on the basis of yield per culture, % wise this showed distinct enhancement and it was 2.2 fold more than the control.

When the cells were grown for 2 weeks in growth medium and then 2 weeks in production medium, slight reduction in growth was observed with distinct rise in total sterol content both on yield per culture basis and % wise, which was maximum (0.305%).

Table : V-4

Influence of two stage culture system on growth and sterol production in suspension culture of Commiphora wightii

Inoculum : 400±30 mg tissue by fresh wt.

Growth medium : MS with Kn 0.1 mg l<sup>-1</sup>, 2,4-D 0.1 mg l<sup>-1</sup>,  
Inositol 100 mg l<sup>-1</sup> 2% sucrose.

Production medium : MS medium without nitrate and phosphate source with X 2.0 nitrate, X 1.5 phosphate, 2,4-D 0.01 mg l<sup>-1</sup>, Kn 0.1 mg l<sup>-1</sup> and sucrose 6%.

Incubation : 4 weeks at 25±2°C (16 h, 1000 lux)

| Growth Med :<br>Production<br>Med (weeks) | Fresh wt.<br>mg/cult. | Dry wt.<br>mg/cult. | Total sterol content |           |
|---|-----------------------|---------------------|----------------------|-----------|
|   |                       |                     | ug/cult.             | % dry wt. |
| 1. 1 : 3                                  | 2462.5<br>(±115.6)    | 62.3<br>(±4.8)      | 182.9<br>(±36.2)     | 0.295     |
| 2. 2 : 2                                  | 7536.8<br>(±180.4)    | 235.4<br>(±13.3)    | 716.7<br>(±98.6)     | 0.305     |
| 3. 3 : 1                                  | 8539.4<br>(±198.6)    | 262.6<br>(±15.8)    | 746.7<br>(±98.1)     | 0.285     |
| 4. 4 : 0                                  | 10105.7<br>(±218.6)   | 302.6<br>(±16.2)    | 401.6<br>(±44.6)     | 0.133     |
| 5. 0 : 4                                  | 502.1<br>(±26.8)      | 14.3<br>(±2.6)      | 42.1<br>(±11.2)      | 0.301     |

The data represents an average of 3 replicates.

Figures in the parenthesis represent standard deviations.

Maximum sterol content yield per culture wise was obtained (746.7  $\mu\text{g}/\text{cult.}$ ) when the cells were grown for 3 weeks in growth medium and one week in production medium. However, % wise, the total sterol content was less than in the previous case. The growth of the cells was also not hampered.

Growing the cells for all the four weeks in production medium, drastically reduced the cell growth. The total sterol content was almost maximum on % wise, but it was almost 10 times less on  $\mu\text{g}/\text{culture}$  basis.

This experiment indicates that the sterol yield was maximum when the cells were grown for 3 weeks in growth medium and then for 1 week in production medium. However, the maximum % of total sterol content was obtained, when the cells were grown for 2 weeks in growth medium and then for 2 weeks in the production medium.

V-2.j Changes in growth and sterol content with passages in suspension culture

Growth rates of tissues or cells as well as content of secondary metabolites are observed to decline as they age in culture.

In the present studies, the callus cultures initiated from stem in late 1982 have undergone 39 transfers to freshly

prepared media at regular intervals of 30 days, whereas the suspension culture has undergone 36 transfers to freshly prepared medium at regular intervals of 20 days. To check if any change has occurred in growth rate and total sterol content, the suspension cultures were assayed after every 5 subcultures for growth and total sterol determination. The data is presented in Table V-5).

The growth of the cells was comparatively less in the first few subcultures. But these newly initiated cultures exhibited higher content of total sterols on % basis (0.137). Distinct enhancement in growth was noticed at 5th subculture which almost remained constant till 15th subculture. The total sterol content slightly dropped at 5th subculture and then almost remained constant till 15th subculture (0.134-0.133 %).

After 20th subculture, the growth of cells exhibited progressive decline both on fresh wt. and dry wt. basis. Similar pattern was observed in total sterol content also. After slight reduction at 15th subculture, the total sterol content registered decline at 20th subculture. Even though on yield per culture basis, the total sterol content declined at 25th and 30th level, on % basis, it almost remained constant at 25th and 30th subcultures.

Table : V-5

Changes in growth and sterol content in serial subcultures of Commiphora wightii suspension cultures

Inoculum : 400±30 mg tissue by fresh wt.

Medium : MS basal medium, with 2% sucrose.

Supplements : Kn - 0.1 mg l<sup>-1</sup>, 2,4-D - 0.1 mg l<sup>-1</sup>  
Inositol - 100 mg l<sup>-1</sup>

Incubation : 24 days at 25±2°C (16 h, 1000 lux)

| Subculture | Fresh wt.<br>mg/cult. | Dry wt.<br>mg/cult. | Total sterol content |           |
|------------|-----------------------|---------------------|----------------------|-----------|
|            |                       |                     | µg/cult.             | % dry wt. |
| 1st        | 4271.5<br>(±165.4)    | 134.2<br>(±10.2)    | 184.5<br>(±26.8)     | 0.137     |
| 5th        | 10108.3<br>(±218.3)   | 310.8<br>(±16.8)    | 417.1<br>(±44.6)     | 0.133     |
| 10th       | 10352.7<br>(±232.4)   | 312.4<br>(±17.1)    | 430.5<br>(±48.2)     | 0.134     |
| 15th       | 10236.8<br>(±214.7)   | 314.2<br>(±15.9)    | 418.2<br>(±38.6)     | 0.133     |
| 20th       | 9846.1<br>(±202.6)    | 308.4<br>(±16.2)    | 382.2<br>(±41.3)     | 0.130     |
| 25th       | 9552.8<br>(±186.4)    | 277.9<br>(±15.8)    | 361.5<br>(±37.8)     | 0.130     |
| 30th       | 9226.4<br>(±170.5)    | 272.1<br>(±14.9)    | 351.5<br>(±35.1)     | 0.130     |

The data represents an average of 3 replicates.

Figures in the parenthesis represent standard deviations.

### V-3 STUDIES ON KETONIC FRACTION AND GUGGULSTERONES

#### V-3.a Comparison of ketonic fraction and Guggulsterones (Z and E) in various materials

Gum-resin of mature trees, growing wild in forest of Vasad was collected as described in Chapter III.1 ( ). Mature leaves and stem explants of the same tree were also collected. Fresh (except gum-resin) and dry weights of these samples were determined. The samples were analyzed for ketonic fraction and Guggulsterones as mentioned in Chapter II-6.a.2.

Table V-6 indicates that the content of ketonic fraction so also guggulsterones was maximum in gum-resin. The percentage of both of these was less in leaf and stem explants. The stem explants contain around 0.06 fold ketonic fraction and guggulsterones, whereas the leaf explants contain around 0.05 fold ketonic fraction and guggulsterones as compared with gum-resin. The percentage was still further reduced in callus initiated from these explants. The callus induced from stem explants exhibited around 0.0043 fold ketonic fraction and guggulsterone as compared with gum-resin and 0.06 fold as compared with the original explant.

Fig. V-9 shows the qualitative analysis by TLC.

Table : V-6

Comparison of ketonic fraction and Guggulsterones (Z and E) in various materials.

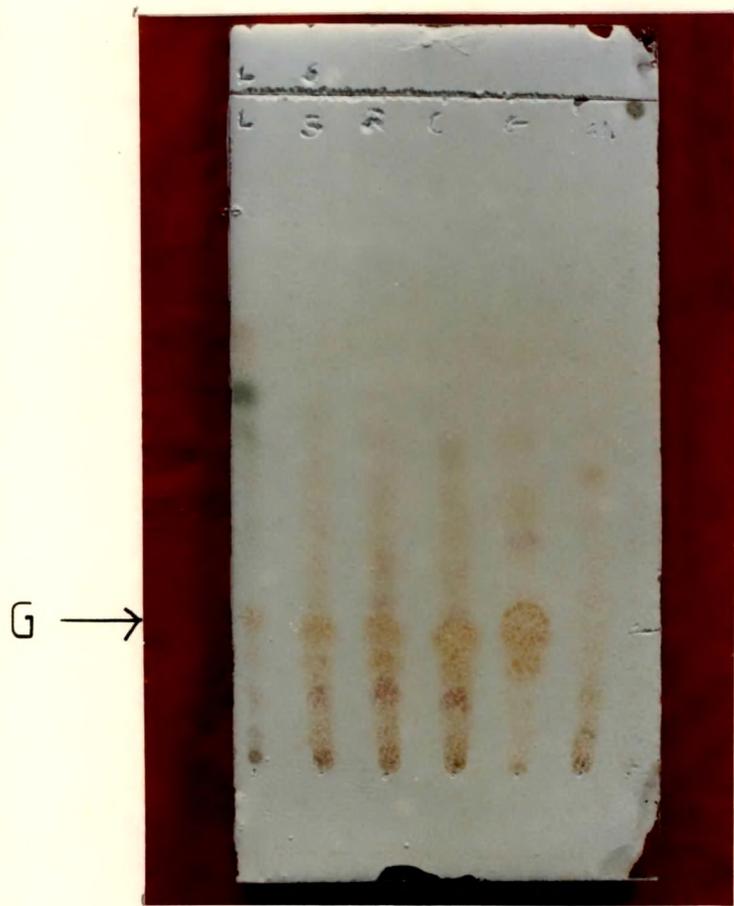
| Material         | Fresh wt.<br>(g) | Dry wt.<br>(g)    | Ketonic<br>fraction | Guggulsterones |
|------------------|------------------|-------------------|---------------------|----------------|
|                  |                  |                   | % of dry wt.        | % of dry wt.   |
| Gum-resin        | -                | 1.0<br>(±0.15)    | 4.32                | 2.1            |
| Stem             | 10.5<br>(±0.42)  | 2.17<br>(±0.20)   | 0.275               | 0.135          |
| Leaf             | 10.2<br>(±0.48)  | 1.86<br>(±0.19)   | 0.235               | 0.116          |
| Callus<br>(Stem) | 10.1<br>(±0.41)  | 0.405<br>(±0.021) | 0.019               | 0.009          |

Data represents an average of 3 replicates.

Figures in parenthesis represent standard deviations.

**Fig. V-9** TLC analysis of ketonic fraction isolated from

- 1 Leaf callus
  - 2 Leaf
  - 3 Stem callus
  - 4 Stem
  - 5 Resin
- G-guggulsterone**



1 2 3 4 5  
Fig. V-9

V-3.b Guggulsterones (Z and E) production of Normal and Ethephon treated suspension cultures of *Commiphora wightii*

The important active principle (guggulsterones Z and E) was found to be present at very low concentrations (0.009%) in callus as well as cell suspension culture. It is known that the  $\beta$ -C-3 sterols act as precursors for guggulsterone and other sterols in *Commiphora wightii* (Bajaj and Sukh Dev, 1982). Thus from a reported linkage between  $\beta$ -C-3 sterols and guggulsterones, we standardized a production medium for  $\beta$ -C-3 sterols which exhibited 2.2 fold increase in the percent total sterol production. However, the high  $\beta$ -C-3 sterol pool did not result in higher pool of guggulsterones. In the following experiments the effect of Ethephon (2-chloroethylphosphonic acid) treatment on guggulsterone production in suspension and callus cultures of *Commiphora wightii* are described.

To conduct these investigations, a suspension culture of *Commiphora wightii* which had been grown for two years in MS medium with  $0.1 \text{ mg l}^{-1}$  of Kn and 2,4-D and 2% sucrose was employed. Ethephon, a commercial preparation containing  $480 \text{ g l}^{-1}$  of 2-chloroethylphosphonic acid was obtained from Agremore Ltd, Bangalore, India. The amount of 2-chloroethylphosphonic acid in various dilutions used in this study will subsequently referred to as active substance. The treatment of active

substance was conducted in four concentrations (1,10,100 and 1,000 mg l<sup>-1</sup> in the medium). While 1,000 mg l<sup>-1</sup> active substance treatment killed all cells, growing cultures were obtained with the lower concentrations of active substance. Both the fresh and dry weights of cultures were reduced with the increasing concentration of active substance, and there was 10.0 fold decrease in fresh wt. and 6.6 fold decrease in dry weight. But the synthesis of guggulsterones was triggered to 0.11% by incorporation of active substance at 100 mg l<sup>-1</sup> concentration in the medium. Thus the guggulsterone content in the ethephon treated cell cultures (active substance, 100 mg l<sup>-1</sup>) exhibited 12.0 fold increase over control (Table V-7).

V-3.c Guggulsterones (Z and E) production in normal and ethephon treated callus cultures

In another experiment ethephon was added on the callus after inoculations at four concentrations (1,10,100 and 1,000 mg l<sup>-1</sup> of active substance). The flasks were covered tightly with aluminum foil. The cells exhibited reduction in growth both on fresh and dry weight basis with absolutely no growth at 1,000 mg l<sup>-1</sup> of active substance. At 100 mg l<sup>-1</sup> concentration of active substance, there was 9.4 fold decrease in fresh wt. and 7.6 fold decrease in dry wt. the production of guggulsterones was enhanced to 0.1% which is 12.5 fold more than the control (Table V-8).

Table : V-7

Guggulsterones (Z and E) production of normal and Ethophen treated suspension cultures of Commiphora wightii.

Inoculum : 400±30 mg tissue by fresh wt.

Medium : MS basal medium with 2% sucrose.

Supplements: Kn - 0.1 mg l<sup>-1</sup>, 2,4-D - 0.1 mg l<sup>-1</sup>  
Inositol - 100 mg l<sup>-1</sup>

Ethephon (active substance) added at different concentrations.

Incubation : 24 days at 25±2°C (16 h, 1000 lux)

| Active substance<br>mg l <sup>-1</sup> | Fresh wt.<br>g/fl. | Dry wt.<br>g/fl.  | Guggulsterones |           |
|--|--------------------|-------------------|----------------|-----------|
|  |                    |                   | ug/cult.       | % dry wt. |
| -                                      | 10.2<br>(±0.37)    | 0.301<br>(±0.02)  | 27.8           | 0.009     |
| 1.0                                    | 7.1<br>(±0.21)     | 0.224<br>(±0.018) | 20.1           | 0.009     |
| 10.0                                   | 4.8<br>(±0.19)     | 0.195<br>(±0.014) | 17.5           | 0.009     |
| 100.0                                  | 1.2<br>(±0.09)     | 0.045<br>(±0.003) | 49.5           | 0.110     |
| 1,000.0                                | 0.45<br>(±0.02)    | 0.028<br>(±0.002) | -              | -         |

Table : V-8

Guggulsterones (Z and E) production of normal and Ethephon treated callus cultures of Commiphora wightii.

- Inoculum : 400±30 mg tissue by fr. wt.  
 Medium : MS basal medium with 2% sucrose.  
 Supplements : Kn - 0.2 mg l<sup>-1</sup>, 2,4-D - 0.2 mg l<sup>-1</sup>  
                   Inositol - 100 mg l<sup>-1</sup>  
 Incubation : 40 days at 25±2°C (16 h, 1500 lux)

| Active substance<br>mg l <sup>-1</sup> | Fresh wt.<br>/cult.<br>(g) | Dry wt.<br>/cult.<br>(g) | Guggulsterones |           |
|--|----------------------------|--------------------------|----------------|-----------|
|  |                            |                          | µg/cult.       | % dry wt. |
| -                                      | 14.2<br>(±0.492)           | 0.650<br>(±0.035)        | 52.2           | 0.008     |
| 1.0                                    | 12.8<br>(±0.442)           | 0.560<br>(±0.031)        | 44.8           | 0.008     |
| 10.0                                   | 5.2<br>(±0.224)            | 0.240<br>(±0.019)        | 21.6           | 0.009     |
| 100.0                                  | 1.5<br>(±0.115)            | 0.085<br>(±0.008)        | 83.5           | 0.10      |
| 1,000.0                                | 0.401<br>(±0.021)          | 0.031<br>(±0.003)        | -              | -         |

Data represents an average of 3 replicates.

Figures in parenthesis represent standard deviations.

Ethephon (active substance) added at different concentrations.