CHAPTER IV - INITIATION AND ESTABLISHMENT OF CALLUS AND SUSPENSION CULTURES

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INITIATION AND ESTABLISHMENT OF CALLUS AND SUSPENSION CULTURES

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INITIATION AND ESTABLISHMENT OF CALLUS AND SUSPENSION CULTURES

Experiments described in this chapter were performed to initiate callus cultures from different parts viz. leaf and stem on a completely defined medium. For designing standard medium, effects of various nutritional and hormonal parameters on callus growth were studied. Experiments were also conducted to initiate fine suspension from the callus culture and examine their growth dynamics.

IV=1 STUDIES WITH CALLUS CULTURES

IV-1.a Initiation of callus culture from leaf and stem explants of Commiphora wightii

In the preliminary studies, three different basal media were tested for callus initiation from leaf and stem explants. Leaf discs and stem explants were inoculated onto White's (1954), MS (1962) and Gamborg's B5 (1968) media (Table IV-1). All these media were supplemented with 2% sucrose and were supplied with the following hormones :-

1. 2,4-Dichlorophenoxyacetic acid (2,4-D) 0.1 mg l^{-1}
2. Kinetin (Kn) 0.1 mg l^{-1}

Though callus initiation occurred on all the three media from the cut surface of explants in the second or third week of the culture, callus tissues grown on these media markedly differed in appearance and texture. The best growth was obtained on MS composition, whereas moderate growth was observed on Gamborg's medium. White's composition showed very poor growth, almost one third of the growth obtained on MS on the dry as well as fresh weight basis. Whitish yellow, friable callus was obtained on MS medium, while the callus tissues initiated on the other two media were brown (Table IV-1; Fig. IV-1).

Table IV-1 Effect of different basal media on the growth of guggul callus.

Supplements : 2,4-D - 0.1 mg l^{-1}
Kn - 0.1 mg l^{-1}
Sucrose - 2%

Incubation : 40 days at 25±2°C (16 h, 1,500 lux)

Basal medium	Inoculum	Fresh wt. mg/cult.		Dry wt. mg/cult.	
MS	Leaf discs	1452.8	(±225.7)	72.8	(±8.2)
Gamborg's	Leaf discs	634.6	(± 40.4)	31.8	(±4.5)
White's	Leaf discs	417.7	(± 31.7)	24.2	(±4.3)
MS	Stem explants	1210.4	(±165.2)	59.6	(±6.8)
Gamborg's	Stem explants	554.2	(± 51.2)	25.8	(±4.8)
White's	Stem explants	433.8	(± 41.2)	22.2	(±4.4)

Data represents an average of 3 replicates.

Figures in the parenthesis represent standard deviation.

Fig. IV-1 Leaf (A) and stem (B) callus of *Commiphora wightii* in MS + Kn (0.2 mg l^{-1}) + 2,4-D (0.2 mg l^{-1}) + Inositol (100 mg l^{-1}).

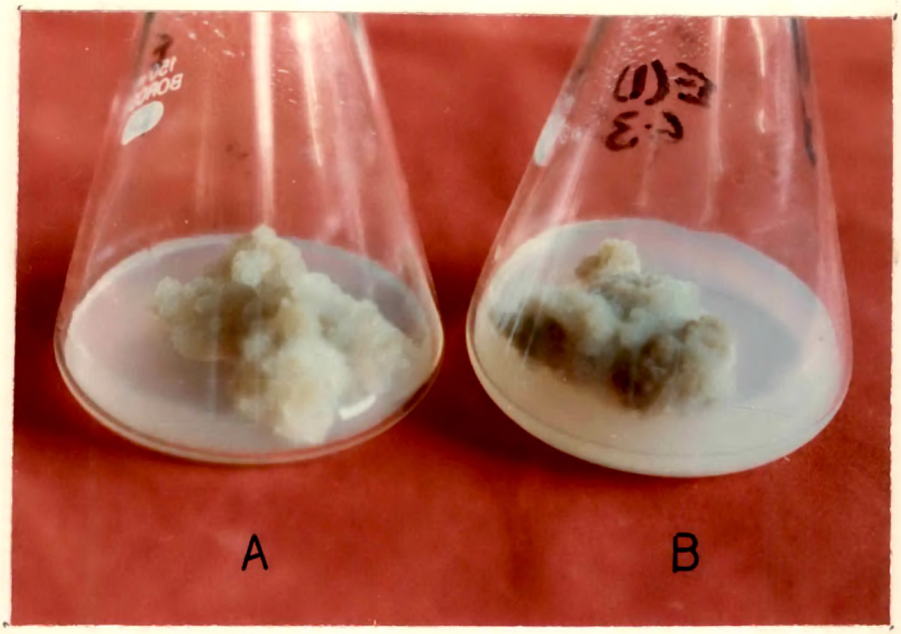

Fig. IV - 1

Since MS composition was found optimal for the callus initiation from both the explants, it was preferred for the subsequent studies.

IV-1.b Effect of auxins on the morphogenetic response of leaf and stem explants

To examine the influence of auxins on the morphogenesis in the explants, 2,4-D, IAA, IBA and ∝NAA were tested over a wide range of concentrations. Each of these auxins were incorporated at 0.1, 0.2, 0.5 and 1.0 mg l^{-1} levels into MS basal medium supplemented with 2% sucrose and Kn 0.1 mg l^{-1}. Leaf and stem explants were prepared as described in Chapter II-3.a.2. Responses of the leaf discs and stem explants observed are presented in Table IV-2.

In 10-15 days of incubation, most of the explants enlarged in size giving a swollen appearance; the midribs in particular swelled showing initiation of callus. Among the four auxins tested, ∝NAA was found to be the most effective in inducing roots. Root induction was observed with all the four concentrations of ∝ NAA from the leaf discs; while, in the case of IAA and IBA, few roots were induced from the leaf discs. With 2,4-D, profuse whitish yellow callus was formed without any morphogenetic response. The stem explants exhibited no rhizogenesis with any of the auxins tested. Shoots too were not induced

Table IV-2 Effect of auxins on the induction of rhizogenesis in leaf explants.

Inoculum : Leaf discs measuring 5 mm in diameter

Medium : MS with Kn 0.1 mg l^{-1} and 2% sucrose.
The auxins were added as indicated below :

Incubation : 40 days at 25±2°C 1,500 lux, 16 h photoperiod.

Auxin	Concentration of auxins (mg l^{-1})	Rooting response	Remarks
IAA	0.1	+	Leaf discs swollen with slight callusing at the midrib and edge of the leaf discs.
	0.2	+	
	0.5	+	
	1.0	-	
IBA	0.1	+	Slight callusing at the edge of the leaf discs.
	0.2	+	
	0.5	+	
	1.0	+	
∝ NAA	0.1	++	Callusing at the edge of the leaf discs and at the midrib.
	0.2	+++	
	0.5	+++	
	1.0	++	
2,4-D	0.1	-	Callusing at the edge of the leaf discs.
	0.2	-	Profuse callusing all over the leaf discs.
	0.5	-	Callusing at the edge of the leaf discs.
	1.0	-	

Roots : -, nil; +, few; ++, many; +++, profuse.

with any of these auxins at the concentrations tested from both the explants.

Further experiments with MS medium, by varying hormonal concentrations revealed that the best growth of the callus was obtained at 0.2 mg l^{-1} of Kn, 0.2 mg l^{-1} 2,4-D in presence of Inositol 100 mg l^{-1}. The stock of callus tissues were therefore, maintained in MS medium containing 2% sucrose, 0.2 mg l^{-1} of 2,4-D and Kn and 100 mg l^{-1} Inositol by regular transfers to freshly made media.

IV-1.c Pattern of callus growth

Callus masses weighing 400±30 mg by fresh weight (24.6±4 mg by dry wt.) were transferred from stock cultures onto 30 ml freshly made standard agar medium. In the present studies 'standard medium' denotes MS medium supplemented with 2% sucrose, 0.2 mg l^{-1}, 2,4-D, 0.2 mg l^{-1} Kn and 100 mg l^{-1} Inositol. Every sixth day, a fixed number of three replicates was harvested and callus growth was measured in terms of fresh and dry weights. Growth of callus, initiated both from stem and leaf, followed a typical sigmoid growth curve (Fig. IV-2.a,b). On fresh and dry weight basis, a distinct and prolonged lag phase was observed for initial 12 days. This was followed by a phase of linear growth till day 30. During this period

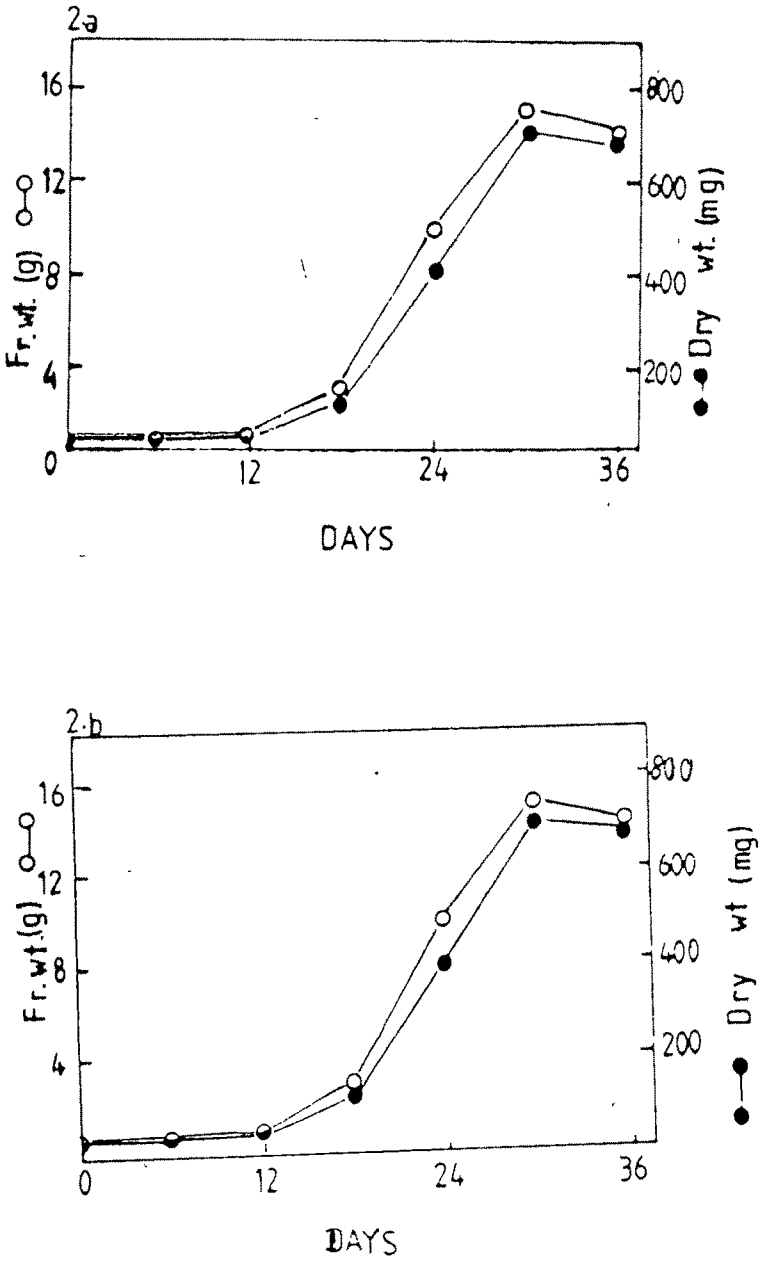

Fig. IV-2 Growth curve of Commiphora wightii callus cultures

2.a Callus initiated from leaf explant

2.b Callus initiated from stem explant

Medium : MS + Kn (0.2 mg l^{-1}) + 2,4-D (0.2 mg l^{-1}) + Inositol (100 mg l^{-1}).

Incubation : 36 days at 25±2°C (16 h, 1000 lux).

there was 37.7 fold increase in fresh wt. and 28.5 fold increase in dry wt. respectively in the case of leaf callus. The callus initiated from stem exhibited 36.5 fold increase in fresh wt. and 28.6 fold increase in dry wt. Thereafter, the growth declined and the callus tended to become hard and brown.

As there was not much difference in the growth of the calli initiated from leaf and stem explants and because the callus derived from stem exhibited higher content of β-C-3 sterols (Chapter V-1) the latter was preferred for further experiments of nutritional requirements.

IV-2 STUDIES ON NUTRITION IN CALLUS CULTURES

Prior to the transfer of tissue on experimental media they were grown on MS basal medium for 8 to 10 days in order to minimize hormonal carryover. Size of the inoculum on experimental medium was 400$\pm$30 mg fresh tissue (corresponding dry wt. was 24.6$\pm$4.0 mg). Tissues were incubated at 25$\pm$2°C and at the end of culture period of 30 days they were analyzed for fresh and dry wt. increases. The results are presented in Tables IV-3 - IV-8.

IV-2.a Effect of different carbon sources on callus growth

The effect of different carbon sources on the growth of callus was tested. Sucrose was omitted from the basal medium and the different carbon sources were added at a concentration of 2%.

As shown in Table IV-3, sucrose and glucose were found to be good supporters of growth on fresh as well as dry wt. basis. Growth was moderate in fructose. The tissue grew poorly on maltose starch, xylose, galactose, lactose and glycerol. The tissue remained pale yellow and friable on sucrose and glucose, while it turned yellowish brown on fructose and maltose and turned hard and brown on all the other carbon sources.

IV-2.b Influence of auxins on callus growth

Auxins IAA, IBA, NAA and 2,4-D were incorporated into the basal medium containing 2% sucrose and 0.1 mg l^{-1} Kn. Each auxin was used at the conc. of 0.1, 0.2, 0.5 and 1.0 mg l^{-1} to study their effect on growth. Tissues grown on basal medium served as control. Cultures were also examined periodically for any morphogenetic response. At the end of incubation of 30 days in culture, the tissues were harvested for the measurement of fresh and dry weights.

Table IV-3 Effect of different carbon sources on growth of guggul callus.

Medium : MS basal medium
Supplements: Kn and 2,4-D at 0.1 mg l^{-1}
all carbon sources added at 2% concentration.
Inoculum : 400±30 mg fresh tissue.
Incubation : 30 days at 25±2°C (16 h photoperiod, 1,500 lux)

Carbon source	Fresh wt. mg/cult.		Dry wt. mg/cult.	
None	413.3	(± 25.1)	21.0	(± 2.7)
Sucrose	12216.8	(±428.3)	572.8	(±29.6)
Glucose	11932.2	(±406.9)	465.2	(±27.2)
Fructose	9826.6	(±302.1)	398.2	(±16.3)
Maltose	912.6	(± 71.6)	52.3	(± 5.4)
Starch	654.3	(± 61.2)	35.2	(± 2.6)
Xylose	630.2	(± 47.2)	31.3	(± 3.1)
Galactose	699.6	(± 60.1)	33.3	(±2.8)
Lactose	626.1	(± 52.2)	34.2	(± 2.7)
Glycerol	405.6	(± 32.9)	22.6	(± 2.8)

Data represents an average of 3 replicates.

Figures in the parenthesis represent standard deviations.

As indicated in Table IV-4, best growth was obtained with 2,4-D followed by ∝ NAA. The growth was maximum at 0.2 mg l^{-1} 2,4-D. While 0.5 to 1.0 mg l^{-1} was supra-optimal for growth.

Of the four levels of ∝ NAA administered, 1.0 mg l^{-1} conc. was optimal for growth. The callus turned compact and green in the presence of ∝ NAA. The other two auxins IAA and IBA showed no significant change/difference compared to the control.

To conclude, of the four auxins tested, 2,4-D at 0.2 mg l^{-1} was most suitable/favourable for callus growth.

IV-2.c Influence of cytokinins

To study the influence of cytokinins on growth, the callus was subjected to 0.1, 0.2, 0.5 and 1.0 mg l^{-1} conc. of kinetin (Kn), 6-benzylaminopurine (BAP) and N^6-γ-γ-dimethylallyl amino purine (2-ip).

Of the four Kn levels, 0.2 mg l^{-1} conc. favoured maximum callus growth, although the callus grew well at 0.1 and 0.5 mg l^{-1} of Kn. Fold wise increases of fresh wt. over the control in four Kn media i.e. 0.1, 0.2, 0.5 and 1.0 mg l^{-1} were X 3.1, X 3.8, X 2.9 and X 1.6 respectively.

BAP also supported callus growth, but growth was

Table IV-4 Effect of auxins on growth of guggul callus

Medium : MS basal medium with 2% sucrose.
Supplements: Kn- 0.1 mg l^{-1} and various auxins at different concentrations.
Inoculum : 400±30 mg callus tissue by fresh wt.
Incubation : 30 days at 25±2°C (16 h, 1,500 lux)

Auxin	Concentration mg l^{-1}	Fresh wt. mg/cult.		Dry wt. mg/cult.	
-	-	1145.2	(± 62.9)	52.5	(± 4.8)
2,4-D	0.1	12493.3	(±446.1)	585.7	(±30.3)
	0.2	13218.2	(±462.3)	653.1	(±32.1)
	0.5	9374.8	(±346.2)	495.5	(±24.8)
	1.0	4057.6	(±283.2)	206.2	(±16.3)
✂ NAA	0.1	3126.2	(±205.2)	142.6	(±10.8)
	0.2	3278.6	(±261.8)	152.8	(±11.3)
	0.5	3826.3	(±270.3)	178.4	(±15.2)
	1.0	4225.6	(±301.2)	291.1	(±18.2)
IAA	0.1	1226.2	(±98.3)	61.1	(± 4.8)
	0.2	1238.6	(±86.2)	62.3	(± 5.1)
	0.5	1078.1	(±68.2)	58.1	(± 4.5)
	1.0	896.2	(±51.2)	35.3	(± 3.9)
IBA	0.1	1878.2	(±108.2)	85.1	(± 6.2)
	0.2	1498.1	(±39.6)	76.5	(± 7.1)
	0.5	1202.5	(±61.8)	56.1	(± 5.6)
	1.0	1115.6	(±49.6)	55.8	(± 5.1)

Data represents an average of 3 replicates.
Figures in the parenthesis represent standard deviation.

markedly lower as compared with Kn. Fold wise increase of fresh wt. over the control in four BAP media i.e. 0.1, 0.2, 0.5 and 1.0 mg l^{-1} were X 1.9, X 2.2, X 1.4 and X 1.01 respectively. No significant difference in growth was registered with 2-ip as compared to the other two cytokinins (Table IV-5).

IV-2.d Influence of different sucrose levels on callus growth

This study was undertaken after optimal hormonal requirement was determined. The sucrose concentration in the standard medium was varied between 1 to 8%.

It is clear from the Table IV-6 that although the tissue grew well on a wide range of sucrose concentrations from 1 to 6%, the best growth was achieved at 2% sucrose. The callus failed to grow and turned brown in absence of sucrose. The water content was more at sucrose levels of 1 to 4% (95%). From 4% onwards there was a progressive decline in the water content because of the high osmotic pressure of the medium (95% to 94%). The callus remained soft, friable and yellowish white at sucrose levels of 1 to 4%. But it tended to turn hard and brown at the sucrose levels over 4%.

Table IV-5 Effect of cytokinins on the growth of guggul callus.

Medium : MS basal medium with 2% sucrose.
Supplements : 2,4-D - 0.2 mg l^{-1} and various cytokinins at different concentrations.
Inoculum : 400±30 mg callus tissue by fresh wt.
Incubation : 30 days at 25±2°C (16 h, 1,500 lux)

Cytokinin	Concentration mg l^{-1}	Fresh wt. mg/cult.	Dry wt. mg/cult.
-	-	3826.1 (±265.2)	183.2 (±18.2)
Kn	0.1	12116.6 (±428.6)	601.8 (±28.2)
	0.2	14628.2 (±472.8)	706.3 (±34.2)
	0.5	11267.1 (±372.1)	553.2 (±28.1)
	1.0	6224.8 (±286.3)	302.3 (±18.9)
BAP	0.1	7628.5 (±291.3)	378.4 (±21.6)
	0.2	8672.2 (±298.5)	443.6 (±22.7)
	0.5	5996.3 (±255.4)	299.8 (±19.8)
	1.0	3872.4 (±201.8)	198.6 (±17.3)
2-ip	0.1	4118.6 (±216.9)	201.9 (±18.1)
	0.2	3926.8 (±195.6)	186.3 (±17.6)
	0.5	2985.3 (±188.7)	148.2 (±10.2)
	1.0	2576.3 (±175.6)	121.3 (± 7.8)

Data represents an average of 3 replicates.

Figures in the parenthesis are standard deviations.

Table IV-6 Effect of varying sucrose levels on growth of guggul callus

Medium : MS basal medium

Supplements : 2,4-D - 0.2 mg l^{-1}, Kn - 0.2 mg l^{-1}

Sucrose added at different levels.

Inoculum : 400±30 mg callus tissue by fresh wt.

Incubation : 30 days at 25±2°C (16 h, 1,500 lux)

Sucrose concentration %	Fresh wt. mg/cult.		Dry wt. mg/cult.	
-	415.6	(±25.3)	19.85	(± 3.4)
1	11215.3	(±386.4)	566.7	(±28.2)
2	14826.3	(±482.6)	741.3	(±33.1)
3	12682.8	(±436.2)	638.2	(±31.9)
4	11892.6	(±364.8)	598.8	(±30.8)
5	8468.2	(±286.2)	440.4	(±23.2)
6	7266.1	(±263.6)	386.0	(±18.6)
7	1883.3	(±125.7)	109.2	(± 6.5)
8	926.4	(± 52.6)	55.5	(± 4.2)

Data represents an average of 3 replicates.

Figures in the parenthesis are standard deviations.

IV-2.e Influence of vitamins

The influence of vitamins was studied by adding vitamins in the medium singly and in combinations at the concentrations stated in the standard MS medium. When added singly, vitamins did not enhance growth of the callus. It is difficult to assess the effect of single vitamins because the control (without vitamins) also showed some growth possibly due to carry-over. However, good growth was registered, when all the three vitamins were pooled at the concentration in the standard medium; thus exhibiting a synergistic effect of all the vitamins (Table IV-7).

IV-2.f Influence of various inositol levels on callus growth

To determine the optimal inositol level on callus growth, the inositol concentration in the standard medium was varied between 20 to 1,000 mg l^{-1}.

It is observed from Table IV-8 that its absence gave slight growth. Though the callus grew well on a wide range of inositol concentrations from 20 to 400 mg l^{-1}, the best growth was exhibited at 200 mg l^{-1} both on fresh and dry wt. basis. Further increase in inositol concentration showed decline in growth. The callus also turned brown and hard at higher concentrations. However, the callus turned more friable by incorporation of extra inositol 100 mg l^{-1} in the medium.

Table IV-7 Effect of vitamins on the growth of guggul callus

Medium : Only mineral salts in MS medium with 2% sucrose.
Supplements : 2,4-D and Kn - 0.2 mg l^{-1}
Vitamins added as indicated below mg l^{-1}
Inoculum : 400±30 mg callus tissue by fresh wt.
Incubation : 30 days at 25±2°C (16 h, 1,500 lux)

Vitamins mg l^{-1}	Fresh wt. mg/cult.		Dry wt. mg/cult.	
-	1429.1	(± 98.6)	73.2	(± 5.8)
Niacin (0.5)	1516.5	(±102.3)	76.1	(± 6.2)
Thiamine HCl (0.1)	2826.3	(±158.2)	146.8	(± 9.8)
Phyridoxine HCl (0.5)	2114.8	(±148.6)	107.7	(± 6.2)
Thiamine HCl (0.1) + Pyridoxine HCl (0.5)	5872.8	(±232.8)	295.6	(±18.6)
Pyridoxine HCl (0.5) + Niacin (0.5)	4226.9	(±213.5)	215.3	(±17.2)
Thiamine HCl (0.1) + Niacin (0.5)	5612.3	(±201.8)	280.8	(±18.6)
Pyridoxine HCl (0.5) + Niacin (0.5) + Thiamine HCl (0.1)	14826.0	(±448.6)	702.3	(±36.2)

Data represents an average of 3 replicates.

Figures in the parenthesis are standard deviations.

Table IV-8 Effect of varying Inositol levels on growth of guggul callus

Medium : MS basal medium with 2% sucrose but no Inositol

Supplements : 2,4-D and Kn - 0.2 mg l^{-1}

Inositol added at different concentrations (20 to 1,000 mg l^{-1})

Inoculum : 400±30 mg callus tissue by fresh wt.

Incubation : 30 days at 25±2°C (16 h, 1,500 lux)

Inositol concentration mg l^{-1}	Fresh wt. mg/cult.	Dry wt. mg/cult.
-	1226.8 (± 88.3)	60.3 (± 5.2)
20	11862.1 (±368.6)	586.6 (±30.1)
100	14428.3 (±465.8)	718.4 (±38.6)
200	15216.1 (±471.2)	758.8 (±39.2)
400	10482.6 (±321.3)	519.1 (±27.8)
800	826.5 (± 61.2)	39.8 (± 4.2)
1,000	512.3 (± 47.2)	25.1 (± 3.8)

Data represents an average of 3 replicates.

Figures in the parenthesis are standard deviations.

Conclusion : The experiments conducted to determine the influence of various parameters on the growth of guggul callus have indicated that the best growth of callus was achieved on MS medium supplemented with 2% sucrose and 100 mg l^{-1} inositol. The hormonal concentration found to be optimal for the growth was 0.2 mg l^{-1} Kn and 0.2 mg l^{-1} 2,4-D.

IV-3 STUDIES WITH SUSPENSION CULTURES

IV-3.a Establishment of suspension culture

The cell suspension was obtained from callus as described in Materials and Methods (Chapter II-3.c). The callus grown on standard medium was very friable and so suspension culture consisting of free cells and cell aggregates was achieved with agitation in the liquid culture medium. Fine, uniform suspension was obtained by filtering through 500/μ^2 nylon mesh. The cell suspension consists of free cells and cell aggregates ranging from 2 to 15 cells/clump (Fig. II- 1). At the time of subculture, the suspension was again filtered through 500/μ^2 nylon mesh. Five ml of the filtrate was inoculated into 50 ml of fresh culture medium. A pipettable cell suspension was thus obtained after at least 4-6 subcultures.

IV-3.b Effect of different concentrations of 2,4-D and Kn on growth of suspension culture

The combinations of the hormones 2,4-D and Kn studied were

		Kn		
		0.1	0.2	0.4
	0.1			
2,4-D	0.2		Control	
	0.4			

The results of the experiment are presented in Table IV-9. Of the various combinations of Kn and 2,4-D studied, 0.1 mg l^{-1} 2,4-D and 0.1 mg l^{-1} Kn favoured maximum growth of cells. So the hormonal concentration for the suspension culture was reduced to 0.1 mg l^{-1} of Kn and 2,4-D. As extra inositol 100 mg l^{-1} increased the friability of the callus, it was routinely incorporated in the suspension culture also.

IV-3.c Growth kinetics of cell suspension culture

About 6 month old cell suspension was used for growth analysis. In the present studies 'Standard medium' denotes MS medium supplemented with 2% sucrose, 0.1 mg l^{-1} Kn and 2,4-D and 100 mg l^{-1} Inositol. 5 ml (400$\pm$30 mg) cell suspension of stationary phase culture was pipetted into

Table IV-9 Influence of auxin 2,4-D and cytokinin Kn on growth in suspension culture of *Commiphora wightii*

Inoculum : 400±30 mg tissue by fresh wt.

Medium : MS basal medium with 2% sucrose.

Supplements : Inositol - 100 mg l^{-1}

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

Incubation : 24 days at 25±2°C (16 h, 1,500 lux)

Additives mg l^{-1}		Fresh wt. mg/cult.		Dry wt. mg/cult.	
Kn	2,4-D				
0.1	0.1	10358.1	(±214.1)	310.1	(±13.5)
0.2	0.1	9269.3	(±201.8)	276.8	(±12.6)
0.4	0.1	8036.5	(±186.8)	258.6	(±10.2)
0.1	0.2	7830.2	(±176.3)	241.8	(±11.6)
0.2	0.2	7246.9	(±169.2)	224.3	(± 9.2)
0.4	0.2	5926.8	(±148.6)	188.7	(±10.9)
0.1	0.4	4684.3	(± 98.4)	145.5	(± 6.8)
0.2	0.4	3578.2	(± 86.3)	112.9	(± 5.8)
0.4	0.4	2284.6	(±59.8)	70.8	(± 4.9)

Data represents an average of 3 replicates.

Figures in the parenthesis represent standard deviations.

30 ml of culture medium. Cells from a fixed number of three replicates were harvested at four days intervals. Growth parameters such as fresh wt., dry wt. and packed cell volume were determined as described in Materials and Methods (Chapter II-4.a,b,c). Of the three parameters of growth viz. fresh wt., dry wt. and packed cell volume per flask exhibited a lag phase of 8 days after fresh inoculation (Fig. IV-3). Thereafter a rapid period of growth commenced which lasted upto day 16. The growth became almost stationary by day 20 during which the suspension was ready for subculture. Thereafter, the growth declined and the suspension turned brown.

During the exponential phase there was 25.7 fold increase in fresh wt., 25 fold increase in dry wt. and 17 fold increase in packed cell volume.

As in the case of growth of tissue, the maximum growth was obtained on 16th day in suspension culture which is only half period as compared with the static cultures. Hence all the subsequent experiments regarding the secondary metabolites were carried out in suspension culture.

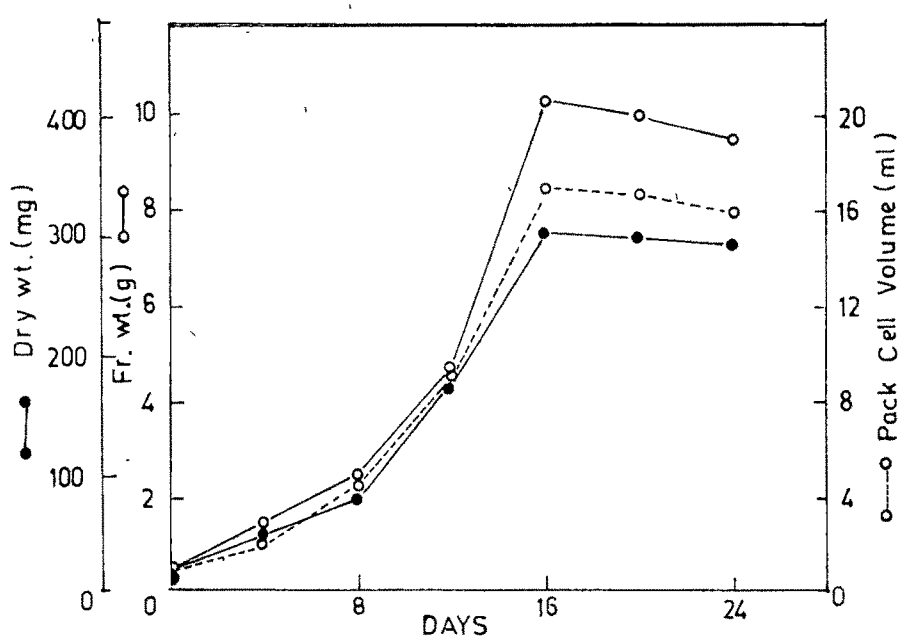

Fig. IV-3 Growth kinetics of cell suspension culture of Commiphora wightii

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

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