CHAPTER IV

STUDIES ON POLYPHENOL SYNTHESIS IN TISSUE CULTURES OF DATURA, AND CASSIA

STUDIES ON POLYPHENOL SYNTHESIS IN TISSUE CULTURES OF DATURA AND CASSIA

The ability of cultured plant tissues to produce secondary metabolites has been reported in a number of cases including Ammi visnaga (Kaul and Staba, 1967), Ruta graveolens (Reinhard et al., 1968), Petroselium hortens (Halbrock and Wellman, 1970), Paul's Scarlet rose (Davies, 1972).

However, the polyphenol production in non-pigmented tissue cultures has received rather less attention. The experiments presented below report the quantitative changes that occur in the total polyphenol content of the batch cultured Datura metel L. cell suspensions and also of the callus cultures derived from Cassia anthers.

The quantitative changes that occur in polyphenol content during the progress of growth in culture as influenced by carbohydrates, nitrates, auxin, kinetin and gibberellic acid (GA_3) were examined.

Experiments were also designed to determine whether physical factors like nature of the medium (i.e. solid or liquid), inoculum size and presence or absence of light alter the polyphenol content in the tissues.

Furthermore, it is reported that light affects the action of growth regulators, thus bringing about physiological and biochemical changes in plant tissues. The effects of light in presence of growth regulators on total phenol production was, therefore, also examined in tissue cultures of Datura.

Finally, the polyphenol production and its relation to induced morphogenetic changes are also examined.

Experiment 4-1 : Effect of Different Sugars on Growth and Polyphenol Production in Datura Cell Suspensions

To find out a suitable carbohydrate source for continuous and rapid growth as well as for enhanced polyphenol production in Datura suspension cultures, measured aliquots of Datura cell suspensions (300±20 mg tissue by fresh weight) were inoculated separately into 150 ml capacity Erlenmeyer flasks containing 40 ml of:-

(i) MS medium with 2% sucrose - Medium A

(ii) " " " 2% glucose - Medium B

(iii) " " " 2% fructose - Medium C

(iv) " " " 1% glucose + 1% fructose - Medium D

(v) MS medium with 2% maltose - Medium E

(vi) " " " 2% starch - Medium F

The flasks were continuously agitated on a horizontal rotary shaker in light at a constant temperature of 26±2°C. A fixed number of replicate flasks of each treatment was harvested after 20 days incubation for the determination of fresh weight, dry weight and total phenol content of the tissues as described in Chapter II, Materials and Methods, 5A, 5B and 6A.

The growth response and the polyphenol accumulation in the cells as influenced by various sugars are presented in Table 7 and illustrated in Fig. 9. The results clearly showed that the starch was the poorest source of carbohydrate for the growth of the cells; while sucrose (Medium A) supported the maximum growth (about 28 fold increase in fresh weight and 19 fold increase in dry weight). Maximum amount of polyphenol accumulation was also registered in the cells grown on medium containing sucrose. Growth attained when glucose (Medium B) and fructose (Medium C) supplied separately was almost similar to that when equimolar mixture of glucose and fructose was added together in medium D (about 21 fold increase in fresh weight and 23 fold increase in dry weight). However, there was considerable increase in

Table 7 : Effect of Different Sugars on Growth and Polyphenol Production in Datura Cell Suspensions*

Inoculum : 300±20 mg tissue by fresh weight in 40 ml of:-

A Modified MS medium supplemented with 2% sucrose
B " " " " " 2% glucose
C " " " " " 2% fructose
D " " " " " 1% glucose+ 1% fructose
E " " " " " 2% maltose
F " " " " " 2% starch

Incubation: 20 days in light at 26±2°C.

Medium	Fresh wt. (mg)	Dry wt. (mg)	Polyphenols (µg/culture)
A	8540 (36)	340 (2.3)	442 (3.1)
B	6306 (42)	225 (2.8)	216 (2.8)
C	6540 (38)	232 (1.6)	196 (2.4)
D	6768 (26)	236 (0.8)	302 (2.6)
E	5680 (22)	196 (1.2)	150 (1.8)
F	2480 (34)	86 (0.6)	92 (1.3)

*Data represent average of six replicates.
Figures in the parenthesis represent standard error.

Fig. 9. Effect of different sugars on growth and polyphenol production in *Datura* cell suspensions.

Treatments: Supplements to modified MS medium (Table 3, Chapter II). 2% sucrose (A), 2% glucose (B), 2% fructose (C), 1% glucose + 1% fructose (D), 2% maltose (E), 2% starch (F).

Inoculum size: 300±20 mg tissue in 40 ml medium.

Experimental details as given in Table 7.

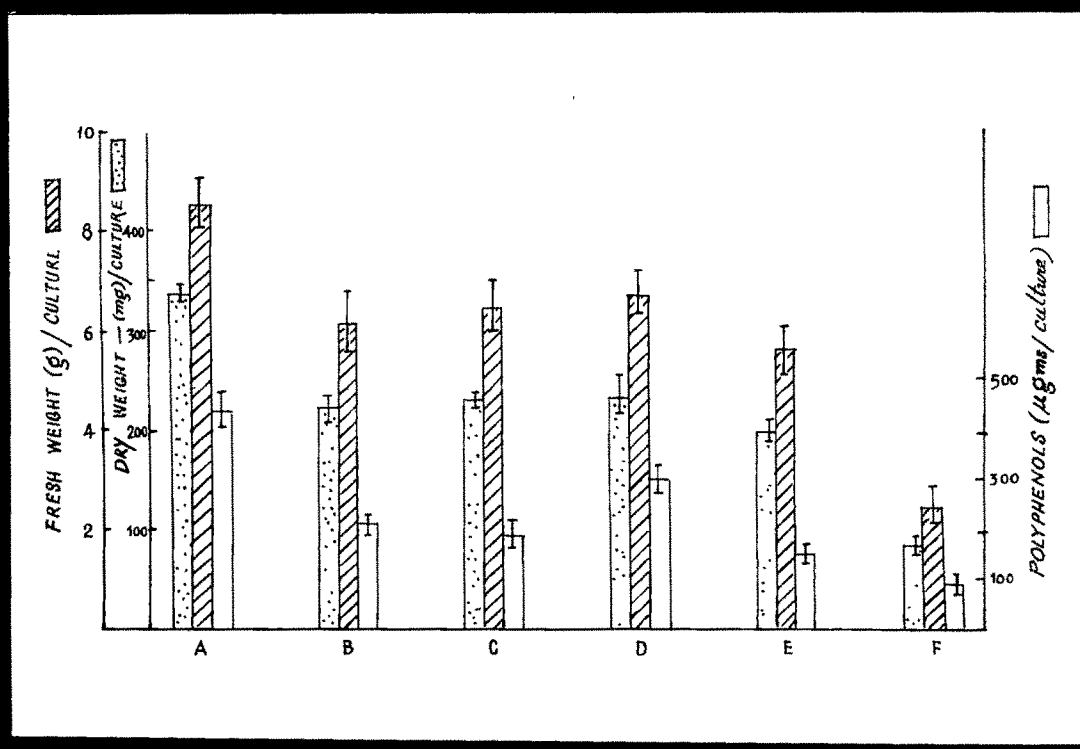
FRESH WEIGHT (g) / CULTURE
DRY WEIGHT (mg) / CULTURE
POLYPHENOLS (µgms / culture)
10
8
6
4
2
400
300
200
100
500
300
100
A
B
C
D
E
F

Fig. 9

the production of total phenols in tissues grown on medium D as compared to that in B and C media which contained glucose or fructose separately. Though better than starch, maltose did not prove to be an efficient carbohydrate source of energy for the rapid growth of the cells and also for the production of polyphenols.

Experiment 4--2 : Effect of Sucrose Concentration on Total Phenol Production in Suspension Cultures of Datura

Modified Murashige and Skoog's medium (Table 3, Chapter II) was supplemented with 0.0, 1.0, 2.0 or 4.0% sucrose in addition to 2.0 mg/l 2,4-D and 0.4 mg/l kinetin to examine the effect of sucrose level on the production of polyphenols in Datura cell suspensions. Measured aliquots of regularly subcultured cell suspensions weighing approximately 300±20 mg tissue by fresh weight, were inoculated into Erlenmeyer flasks containing 40 ml medium. The flaks were incubated on a rotary shaker in light at a constant temperature of 26±2°C.

A fixed number of replicate flasks was harvested at intervals of five days upto 20 days, for the extraction and estimation of total phenols as described in Chapter II, Materials and Methods, 6A.

The polyphenol accumulation at different sucrose levels, as shown in Fig. 10 and Table 8, clearly indicated that increase in sucrose content of the medium led to increase in polyphenol accumulation. The maximum rate of polyphenol accumulation was recorded at 4% sucrose level on day 15. Clearly, the results recorded in the present experiment showed that the total phenol production was dependent on the availability of carbohydrate. Further, the period of maximum polyphenol production preceded the phase of most rapid growth of the tissue.

The pattern of accumulation of phenolic compounds in relation to growth of the tissue at 2% sucrose which was optimal for growth is shown in Fig. 11. During the lag phase of the growth cycle, which extended upto day 5, there was no appreciable change in the polyphenol content of the cells. However, during the pre-exponential phase which extended from 5 to 10 days, there was a very rapid and significant increase in total phenols. During the

Table 8 : Effect of Sucrose Concentrations on Total Phenol Production in Suspension Cultures of *Datura**

Inoculum : 300±20 mg tissue by fresh weight in 40 ml of modified MS medium (Table 3, Chapter II) supplemented with 0.0, 1.0, 2.0 or 4.0 per cent sucrose.

Incubation: 20 days in light at 26±2°C.

Time (days)	Sucrose Concentration (%)							
	0.0		1.0		2.0		4.0	
	Fresh wt. (mg)	Polyphenols (µg/culture)	Fresh wt. (mg)	Polyphenols (µg/culture)	Fresh wt. (mg)	Polyphenols (µg/culture)	Fresh wt. (mg)	Polyphenols (µg/culture)
0	300±20 (6)	37 (0.8)	300±20 (6.)	37 (0.8)	300±20 (6)	37 (0.8)	300±20 (6)	37 (0.8)
5	378 (6)	90 (1.2)	554 (10)	100 (2.1)	482 (12)	32 (0.8)	450 (8)	150 (1.5)
10	512 (8)	120 (0.6)	680 (12)	300 (1.2)	963 (16)	421 (1.3)	932 (12)	550 (1.2)
15	2626 (25)	80 (0.8)	4132 (36)	312 (1.4)	8017 (52)	480 (1.6)	7020 (50)	600 (1.6)
20	2601 (24)	50 (0.6)	4501 (38)	302 (1.2)	8460 (48)	460 (1.1)	9502 (52)	580 (2.2)

*Data represent average of six replicates.
Figures in the parenthesis represent standard error.

Fig. 10. Changes in polyphenol content of Datura cells grown in presence of sucrose at the following Concentrations: 0%; 1%; 2%; 4%.

Inoculum size: 300±20 mg tissue in 40 ml of modified MS medium Table 3, Chapter II).

Experimental details as given in Table 8.

Fig. 11. Progressive changes in polyphenol content of Datura cells in relation to growth (Fr. Wt.).

Inoculum size: 300±20 mg tissue in 40 ml of modified MS medium (Table 3, Chapter II) supplemented with 2% sucrose, 2.0 mg/l 2,4-D and 0.4 mg/l kinetin.

Experimental details as given in Table 8.

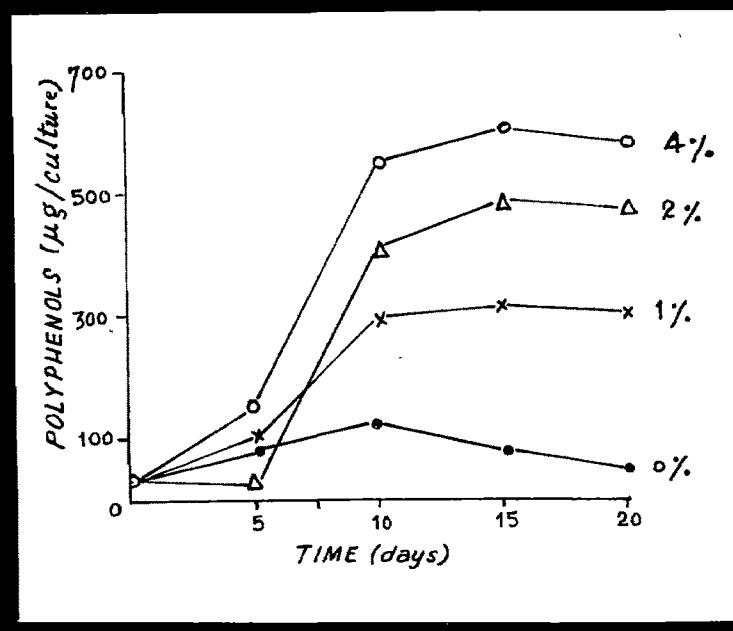
POLYPHENOLS (µg/culture)
700
500
300
100
0
5
10
15
20
TIME (days)
4%
2%
1%
0%

Fig. 10

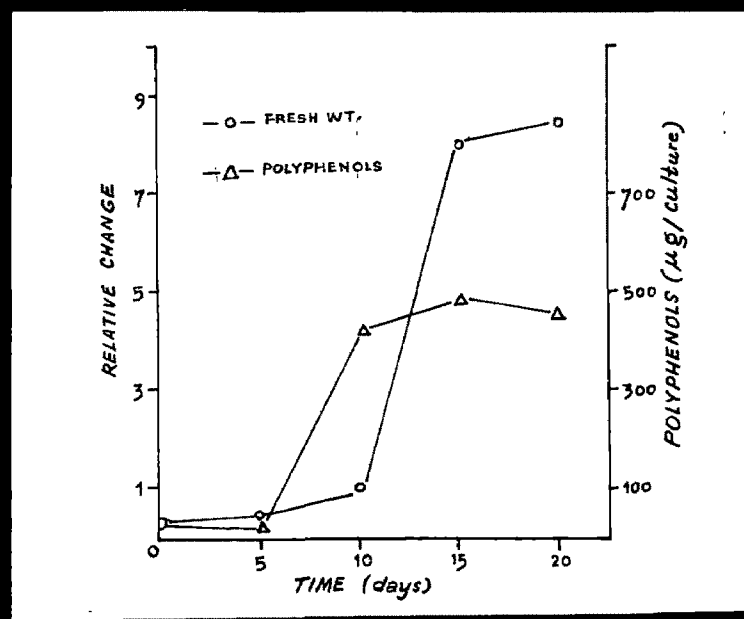
RELATIVE CHANGE
9
7
5
3
1
0
5
10
15
20
TIME (days)
—o— FRESH WT.
—Δ— POLYPHENOLS
POLYPHENOLS (µg/culture)
700
500
300
100

Fig. 11

subsequent period (10 to 15 days) of most pronounced growth the polyphenol production declined.

Experiment 4-3 : Effect of 2,4-dichlorophenoxyacetic Acid on the Production of Polyphenols in Datura Cell Suspensions

Measured aliquots of cell suspension weighing approximately 300±20 mg tissue by fresh weight were transferred to 40 ml modified MS medium (Table 3, Chapter II) supplemented with 0, 10^{-6}, 10^{-5}, $2x10^{-5}$ or $5x10^{-5}$M 2,4-D in presence of 2% sucrose and 0.4 mg/l kinetin, to determine the effect of auxin on polyphenol production. The culture vessels were incubated under conditions mentioned in previous experiments and a fixed number of replicates was harvested at 5 days interval upto 20th day for the measurement of fresh weights and for the estimation of total phenols.

The effect of 2,4-D concentrations on polyphenol accumulation and on gfowth in Datura cells is shown in Fig. 12, 13 and Table 9. The results clearly showed that with the increase in the concentration of auxin there was considerable delay in the initiation of polyphenol synthesis; the synthesis being quite marked in absence of 2,4-D during the initial 5 days which constituted the

Table 9 : Effect of 2,4-dichlorophenoxyacetic acid on the Production of Polyphenols in *Datura* Cell Suspensions*

Inoculum : 300±20 mg tissue by fresh weight in 40 ml of modified MS medium (Table 3, Chapter II) supplemented with 0, 10^{-6}, 10^{-5}, $2x10^{-5}$ or $5x10^{-5}$M 2,4-D.

Incubation: 20 days in light at 26±2°C.

Time (days)	2,4-D Concentrations (M)									
	0		10^{-6}		10^{-5}		$2x10^{-5}$		$5x10^{-5}$	
	Fresh wt. (mg)	Polyphenols (µg/culture)	Fresh wt. (mg)	Polyphenols (µg/culture)	Fresh wt. (mg)	Polyphenols (µg/culture)	Fresh wt. (mg)	Polyphenols (µg/culture)	Fresh wt. (mg)	Polyphenols (µg/culture)
0	300±20 (5)	36 (0.4)	300±20 (5)	36 (0.4)	300±20 (5)	36 (0.4)	300±20 (5)	36 (0.4)	300±20 (5)	36 (0.4)
5	480 (8)	70 (0.8)	402 (10)	30 (0.8)	484 (13)	25 (0.8)	508 (10)	32 (0.4)	378 (8)	22 (0.2)
10	902 (18)	104 (1.6)	941 (16)	202 (1.2)	960 (18)	422 (1.2)	864 (20)	310 (2.6)	418 (12)	262 (2.3)
15	1152 (22)	128 (1.8)	5020 (12)	218 (2.8)	8107 (50)	486 (2.1)	3746 (38)	336 (2.0)	2070 (26)	281 (1.8)
20	1163 (26)	130 (2.2)	5630 (25)	224 (2.6)	8492 (48)	468 (2.0)	3752 (42)	320 (2.4)	2086 (30)	284 (1.2)

*Data represent average of six replicates.
Figures in the parenthesis represent standard error.

Fig. 12. Changes in polyphenol content of Datura cells grown in presence of 2,4-D at the following concentrations: 0 M; 10^{-6}M; 10^{-5}M; $2x10^{-5}$M; $5x10^{-5}$M.

Inoculum size: 300±20 mg tissue in 40 ml of modified MS medium (Table 3, Chapter II).

Experimental details as given in Table 9.

Fig. 13. Effect of 2,4-D concentrations on growth of Datura cell suspensions.

Inoculum size: 300±20 mg tissue in 40 ml of modified MS medium supplemented with 0, 10^{-6}, 10^{-5}, $2x10^{-5}$ or $5x10^{-5}$M 2,4-D.

Experimental details as given in Table 9.

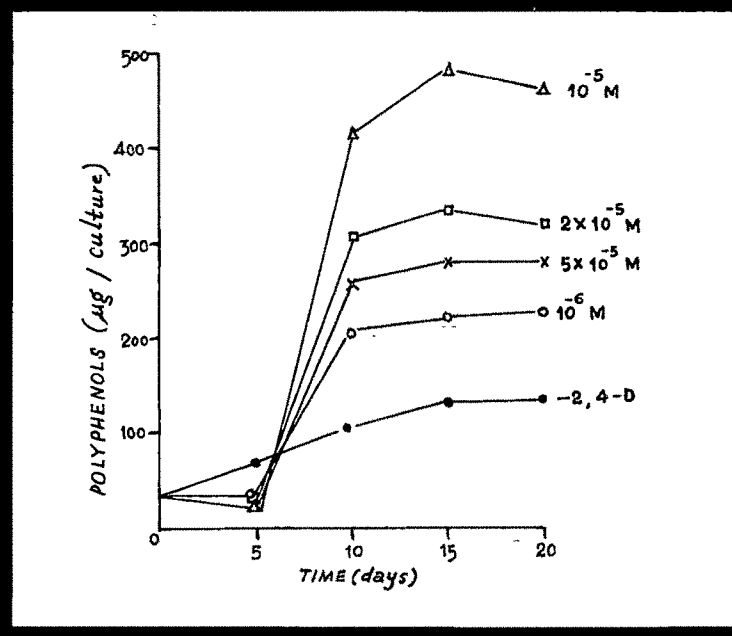
POLYPHENOLS (µg / culture)
500
400
300
200
100
0
5
10
15
20
TIME (days)
10^-5 M
2x10^-5 M
5x10^-5 M
10^-6 M
-2,4-D

Fig. 12

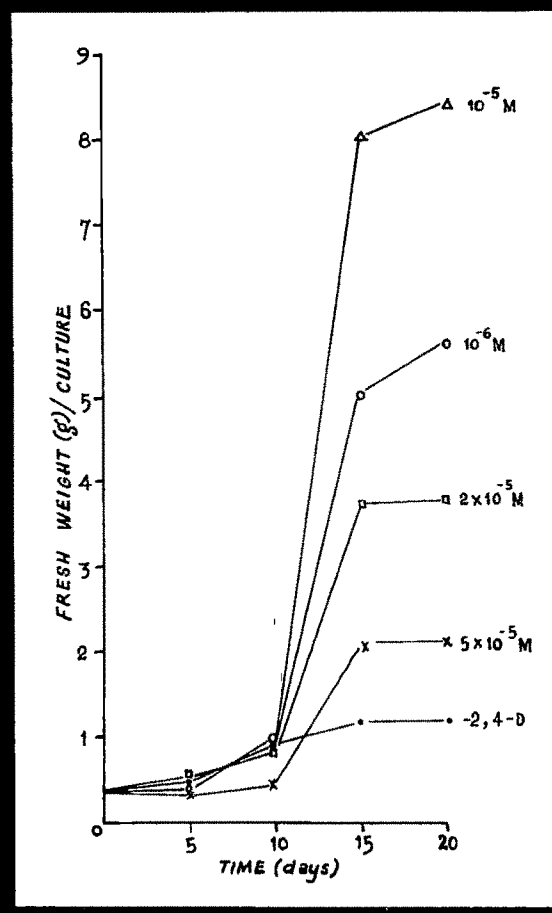
FRESH WEIGHT (g)/ CULTURE
9
8
7
6
5
4
3
2
1
0
5
10
15
20
TIME (days)
10^-5 M
10^-6 M
2x10^-5 M
5x10^-5 M
-2,4-D

Fig. 13

lag phase of growth. However, the accumulation of polyphenols was most pronounced during the period of 5 to 10 days - i.e. the pre-exponential phase of growth at all the concentrations of 2,4-D tested. At the time when growth of the cells was most rapid between 10 and 15 days, there was no further pronounced increase in their polyphenol content. Further, the results also showed that with the increase in the concentration of 2,4-D, there was decrease in the rate of polyphenol production per unit fresh weight. The maximum synthesis of polyphenols was recorded at 10^{-5}M 2,4-D concentration which also supported the maximum growth of the cells (Experiment 3-1, Chapter III).

Experiment 4-4 : Effect of Kinetin on the Synthesis of Polyphenols in Datura Cell Suspension Cultures

Measured aliquot of cell suspensions weighing approximately 300±20 mg tissue by fresh weight, were transferred to Erlenmeyer flasks containing 40 ml of the defined medium (Table 3, Chapter II) supplemented with 0, 10^{-6}, $2x10^{-6}$, 10^{-5} or $2x10^{-5}$M kinetin in presence of 2% sucrose and 2.0 mg/l 2,4-D. The culture flasks were incubated under conditions as mentioned earlier and

replicate number of flasks was harvested at the interval of five days upto 20 days for determining fresh weights and for the estimation of total phenols.

The results obtained are presented in Table 10 and illustrated in Fig. 14 and 15. Unlike auxin, kinetin at highest concentration tested enhanced polyphenol production during the initial 5 days (Fig. 14). Further, in presence of optimal concentration (2.0 mg/l) of 2,4-D, the higher concentrations of kinetin did not supress the polyphenol production; whereas, as noted in previous Experiment (4-2), in presence of optimal concentration of kinetin supra-optimal levels of 2,4-D supressed polyphenol production.

As shown in Fig. 14 and 15 and Table 10, the maximum production of polyphenols was restricted to pre-exponential phase (5 to 10 days period) in all the concentrations tested. During the subsequent period of most rapid growth (10 to 15 days) the polyphenol accumulation continued; it being most pronounced in absence of kinetin. In stationary phase of growth there was registered further increase in polyphenol content in tissues cultured in supra-optimal level. The maximum synthesis of polyphenols, as recorded on day 15, was achieved at 2×10^{-6} M kinetin concentration which also supported the maximum growth of cells in culture (Experiment 3-1, Chapter III).

Table 10 : Effect of Kinetin on Growth and Polyphenol Production in *Datura* Cell Suspensions*

Inoculum : 300±20 mg tissue by fresh weight in 40 ml of modified Murashige and Skoog's medium (Table 3, Chapter II) supplemented with 0, 10^{-6}, $2x10^{-6}$, 10^{-5} or $2x10^{-5}$ M Kinetin.

Incubation: 20 days in light at 26±2°C

Time (days)	Kinetin concentrations (M)									
	0		10^{-6}		$2x10^{-6}$		10^{-5}		$2x10^{-5}$	
	Fresh wt. (mg)	Polyphenols (µg/culture)	Fresh Wt. (mg)	Polyphenols (µg/culture)	Fresh wt. (mg)	Polyphenols (µg/culture)	Fresh wt. (mg)	Polyphenols (µg/culture)	Fresh wt. (mg)	Polyphenols (µg/culture)
0	300±20 ($\bar{6}$)	36 (0.5)	300±20 ($\bar{6}$)	36 (0.5)	300±20 ($\bar{6}$)	36 (0.5)	300±20 ($\bar{6}$)	36 (0.5)	300±20 ($\bar{6}$)	36 (0.5)
5	465 (10)	30 (0.4)	476 (6)	25 (0.6)	462 (13)	30 (0.4)	480 (20)	48 (0.5)	372 (12)	37 (0.4)
10	502 (16)	48 (2.6)	643 (15)	251 (1.9)	963 (18)	420 (3.2)	743 (18)	221 (2.3)	542 (14)	180 (0.8)
15	1102 (32)	154 (1.2)	4302 (48)	265 (2.6)	8602 (50)	480 (2.8)	2806 (38)	370 (2.2)	2502 (56)	315 (2.0)
20	1202 (26)	180 (0.8)	4516 (36)	240 (2.0)	8917 (48)	450 (3.1)	2926 (42)	422 (2.8)	2526 (42)	370 (0.6)

*Data represent average of six replicates.
Figures in the parenthesis represent standard error.

Fig. 14. Changes in polyphenol content of *Datura* cells grown in presence of kinetin at the following concentrations: 0 M; 10^{-6}M; $2x10^{-6}$M; 10^{-5}M; $2x10^{-5}$M.

Inoculum size: 300±20 mg tissue in 40 ml of modified MS medium.

Experimental details as given in Table 10.

Fig. 15. Effect of kinetin on growth of *Datura* cell suspensions.

Inoculum size: 300±20 mg tissue in 40 ml of modified MS medium supplemented with 0, 10^{-6}, $2x10^{-6}$, 10^{-5} or $2x10^{-5}$M kinetin.

Experimental details as given in Table 10.

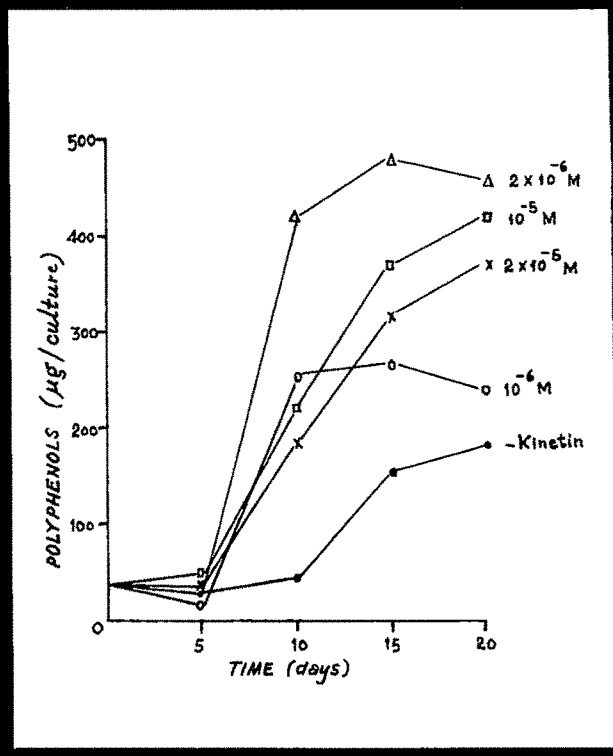
500
400
300
200
100
0
POLYPHENOLS (µg/culture)
5
10
15
20
TIME (days)
Δ 2 x 10⁻⁶ M
□ 10⁻⁵ M
x 2 x 10⁻⁵ M
o 10⁻⁶ M
• Kinetin

Fig. 14

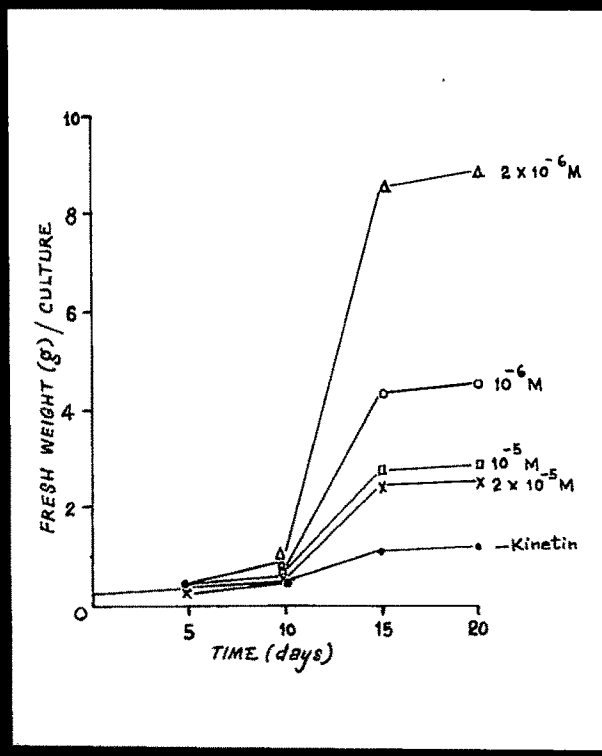
10
8
6
4
2
0
FRESH WEIGHT (g) / CULTURE
5
10
15
20
TIME (days)
Δ 2 x 10⁻⁶ M
o 10⁻⁶ M
□ 10⁻⁵ M
x 2 x 10⁻⁵ M
• Kinetin

Fig. 15

Experiment 4-5 : Effect of Different Sources of Nitrogen on Growth and Polyphenol Synthesis in Suspension Cultures of Datura

To determine the ability of cell suspensions of Datura metel L. in utilizing different sources of nitrogen for the growth of the cells and for total polyphenol production, measured aliquots of cell suspension weighing approximately 300±30 mg tissue by fresh weight were separately inoculated into the Erlenmeyer flasks containing 40 ml of modified Murashige and Skoog's medium (Table 3., Chapter II) supplemented with the following sources of nitrogen:

(i)	MS medium with standard nitrate supply	- Medium A
(ii)	" " without nitrate	- Medium B
(iii)	" " with potassium nitrate	- Medium C
(iv)	" " with Ammonium nitrate	- Medium D
(v)	" " with 2 x Ammonium nitrate	- Medium E
(vi)	" " with 500 mg/l casein hydrolysate	- Medium F
(vii)	" " with 1.0 g/l casein hydrolysate	- Medium G

Polyphenol content and growth responses of cell suspensions, incubated for 30 days in light at 26±2°C to various sources of nitrogen are presented in Table 11 and Fig. 16.

Table 11 : Effect of Nitrogen Source on Growth and Polyphenol Synthesis in Suspension Cultures of *Datura**

Inoculum : 300±30 mg tissue by fresh weight in 40 ml of :-

- A Modified MS medium with standard nitrate supply
- B Modified MS medium without nitrates
- C Modified MS medium with potassium nitrate
- D Modified MS medium with Ammonium nitrate
- E Modified MS medium with 2X Ammonium nitrate
- F Modified MS medium with 500 mg/l casein hydrolysate
- G Modified MS medium with 1.0 g/l casein hydrolysate

Incubation: 30 days at 26±2°C in light,

Medium	Fresh wt. (mg)	Dry wt. (mg)	Polyphenols (µg/culture)
A	8460 (52)	352 (2.0)	460 (2.2)
B	2561 (25)	154 (2.6)	140 (1.8)
C	5021 (68)	218 (3.4)	200 (2.3)
D	6271 (42)	182 (1.8)	250 (2.0)
E	6856 (72)	341 (3.8)	175 (1.4)
F	6700 (48)	285 (2.6)	425 (4.1)
G	6219 (38)	231 (1.8)	445 (3.8)

*Data represent average of six replicates.
Figures in the parenthesis represent standard error.

Fig. 16. Effect of different sources of nitrogen on growth and polyphenol synthesis in *Datura* cell suspensions.

Inoculum size: 300±30 mg tissue in 40 ml modified MS medium supplemented with NH_4NO_3 and KNO_3 (A), without nitrates (B), KNO_3 alone (C), NH_4NO_3 alone (D), 2 x NH_4NO_3 (E), 500 mg/l casein hydrolysate (F), 1.0 g/l casein hydrolysate (G).

Experimental details as given in Table 11.

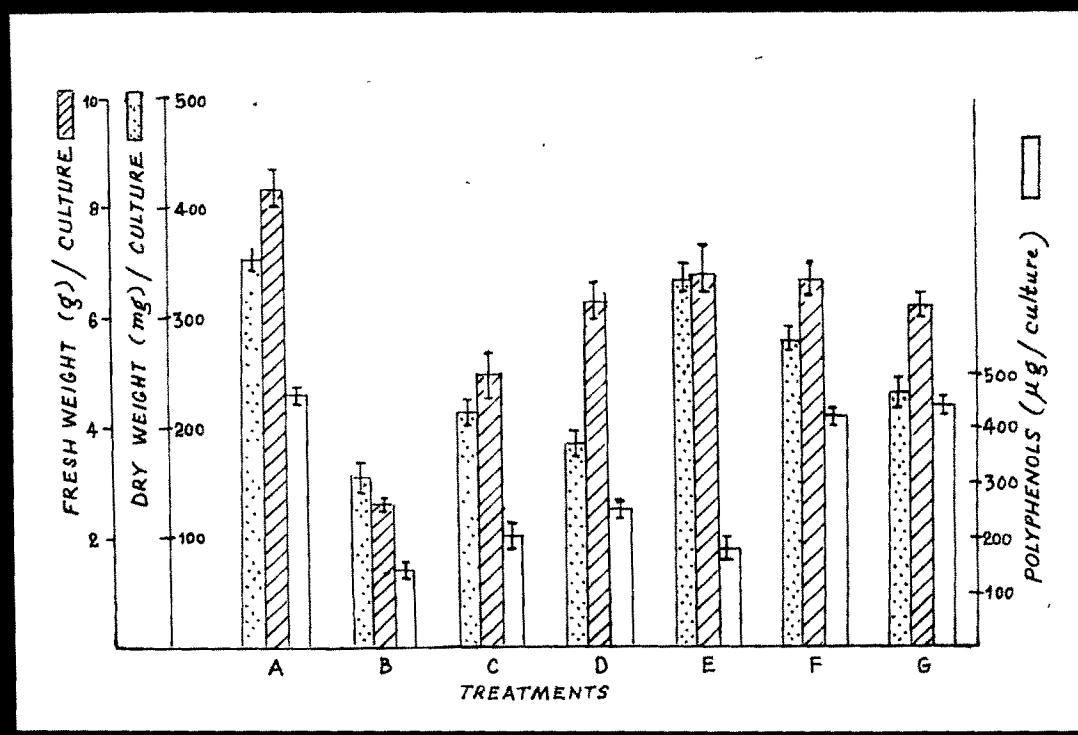
FRESH WEIGHT (g) / CULTURE
DRY WEIGHT (mg) / CULTURE
POLYPHENOLS (µg / culture)
10
8
6
4
2
500
400
300
200
100
500
400
300
200
100
A
B
C
D
E
F
G
TREATMENTS

Fig. 16

The cells cultured on the medium containing both ammonium nitrate and potassium nitrate as nitrogen sources (Medium A) showed maximum accumulation of polyphenols per culture (460 µg/culture) and also maximum growth as determined by fresh weights (28 fold increase) and dry weights (19 fold increase). Double the level of ammonium nitrate (Medium E) proved to be superior to potassium nitrate alone (Medium C) and also casein hydrolysate (Medium F and G) as sole nitrogen source ~~of~~ for growth. However, there was marked reduction in polyphenol accumulation (175 µg/culture) at 2 fold ammonium nitrate level. Of the individual nitrates tested in single dose, ammonium nitrate proved to be considerably superior than potassium nitrate for polyphenol production. Addition of casein hydrolysate as organic source of nitrogen also enhanced the polyphenol accumulation at both the concentrations tested. However, the results indicated that a balanced supply of inorganic nitrogen containing potassium and ammonium nitrates resulted in enhanced accumulation of total phenols in Datura cell suspensions.

Experiment 4-6 : Effect of L-Phenylalanine on Growth and Polyphenol Synthesis in Datura Cell Suspensions

To examine the ability of Datura cell suspensions in utilizing different concentrations of L-phenylalanine for the synthesis of total polyphenols, measured aliquots of cell suspension weighing 300±20 mg tissue by fresh weight were inoculated into Erlenmeyer flasks containing 40 ml of standard culture medium (Table 3, Chapter II) supplemented with 0, 10^{-5}, $5x10^{-5}$, 10^{-4}, $5x10^{-4}$M L-Phenylalanine in addition to 2% sucrose, 2.0 mg/l 2,4-D and 0.4 mg/l kinetin.

The culture flasks were incubated for 30 days as described earlier and a suitable number of replicates was harvested for the determination of fresh and dry weights and for the estimation of total phenols.

The results presented in Table 12 and Fig. 17 clearly indicated that the tissues grown in standard medium (control) had higher polyphenol content than those grown in presence of low doses of L-phenylalanine. The highest concentration of L-phenylalanine tested was found to supress growth of the cells, but promoted polyphenol production. The low concentrations of L-phenylalanine on the other hand, had stimulator effect on

Table 12 : Effect of L-Phenylalanine on Growth and Polyphenol Synthesis in Datura Cell Suspensions*

Inoculum : 300±20 mg tissue by fresh weight in 40 ml of modified MS medium (Table 3, Chapter II) supplemented with 0, 10^{-5}, $5x10^{-5}$, 10^{-4} or $5x10^{-4}$M L-phenylalanine in addition to 2% sucrose, 2.0 mg/l 2,4-D and 0.4 mg/l kinetin.

Incubation: 30 days in light at 26±2°C.

L-Phenylalanine (M)	Fresh wt. (mg)	Dry wt. (mg)	Polyphenols (µg/culture)
0	8602	356	465
	(48)	(2.6)	(2.8)
10^{-5}	12042	379	362
	(74)	(6.6)	(3.1)
5×10^{-5}	6399	326	376
	(68)	(4.4)	(1.8)
10^{-4}	5066	253	385
	(84)	(8.3)	(2.2)
5×10^{-4}	3258	98	597
	(36)	(1.2)	(4.4)

*Data represent average of six replicates.
Figures in the parenthesis represent standard error.

Fig. 17. Effect of L-Phenylalanine on growth and polyphenol synthesis in *Datura* cell suspension cultures.

Inoculum size: 300±20 mg tissue in 40 ml of modified MS medium supplemented with 0, 10^{-5}, $5x10^{-5}$, 10^{-4} or $5x10^{-4}$M L-phenylalanine.

Experimental details as given in Table 12.

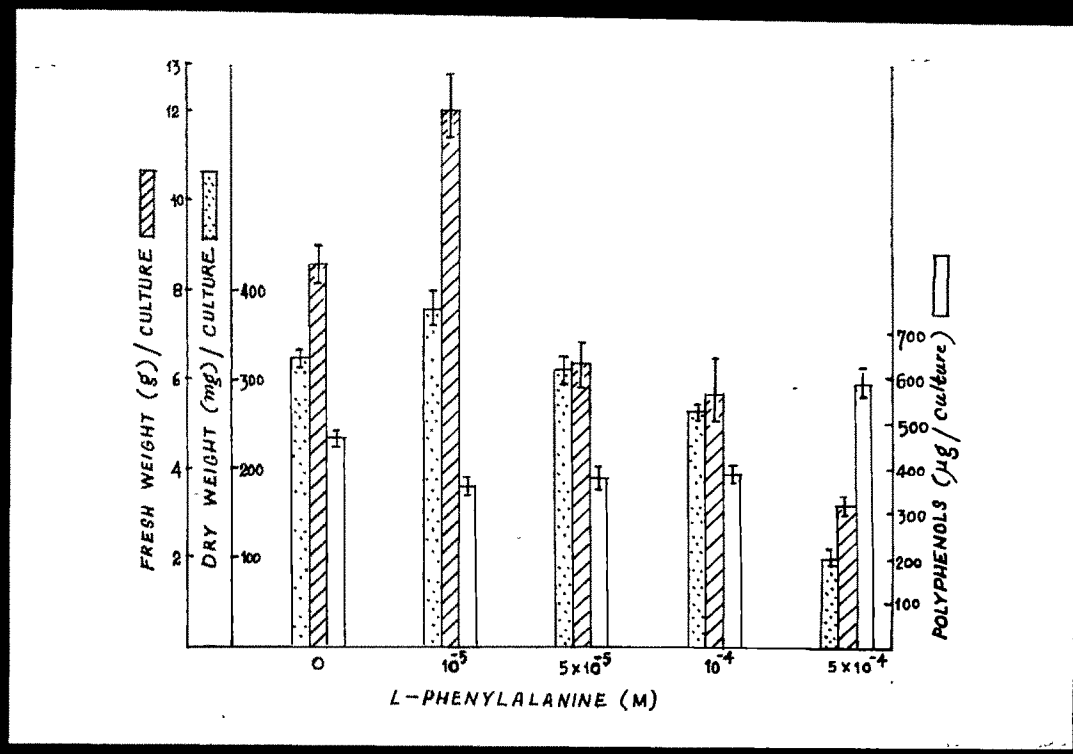
FRESH WEIGHT (g) / CULTURE
DRY WEIGHT (mg) / CULTURE
POLYPHENOLS (µg / culture)
L-PHENYLALANINE (M)
0
10⁻⁵
5x10⁻⁵
10⁻⁴
5x10⁻⁴

Fig. 17

growth of the tissues without influencing significantly polyphenol synthesis.

Experiment 4-7 : Effect of L-Tyrosine on Growth and Total Phenol Production in Datura Cell Suspensions

The experiment described below was performed to examine the effect of different concentrations of L-tyrosine on growth and on the production of polyphenols in Datura cell suspension cultures.

The defined culture medium (Table 3, Chapter II) was supplemented with 0, 10^{-5}, $5x10^{-5}$, 10^{-4} or $5x10^{-4}$M L-tyrosine in addition to 2% sucrose, 2.0 mg/l 2,4-D and 0.4 mg/l kinetin. Cell suspensions weighing 300±20 mg tissue by fresh weight were inoculated to each flask containing 40 ml of medium. The culture flasks were incubated under uniform conditions of light and temperature and a replicate number of flasks was harvested after 30 days for estimating the total phenols accumulated and for determining growth of the tissue.

The data, presented in Table 13 and illustrated in Fig. 18 clearly showed that the addition of L-tyrosine did not enhance the production of polyphenols when compared

Table 13 : Effect of L-Tyrosine on Growth and Total Phenol Production in *Datura* Cell Suspensions*

Inoculum : 300±20 mg tissue by fresh weight in 40 ml of modified MS medium (Table 3, Chapter II) supplemented with 0, 10^{-5}, $5x10^{-5}$, 10^{-4} or $5x10^{-4}$M L-tyrosine in addition to 2% sucrose, 2.0 mg/l 2,4-D and 0.4 mg/l kinetin.

Incubation: 30 days in light at 26±2°C.

L-Tyrosine (M)	Fresh wt. (mg)	Dry wt. (mg)	Polyphenols (µg/culture)
0	8682 (48)	352 (8.6)	465
10^{-5}	10996 (78)	337 (6.2)	275
$5x10^{-5}$	10090 (56)	307 (6.0)	385
10^{-4}	9879 (42)	325 (8.8)	395
$5x10^{-4}$	2142 (18)	65 (2.2)	422

*Data represent average of six replicates.
Figures in the parenthesis represent standard error.

Fig. 18. Effect of L-Tyrosine on growth and polyphenol synthesis in *Datura* cell suspensions.

Inoculum size: 300±20 mg tissue in 40 ml of modified MS medium supplemented with 0, 10^{-5}, $5x10^{-5}$, 10^{-4} or $5x10^{-4}$M L-tyrosine.

Experimental details as given in Table 13.

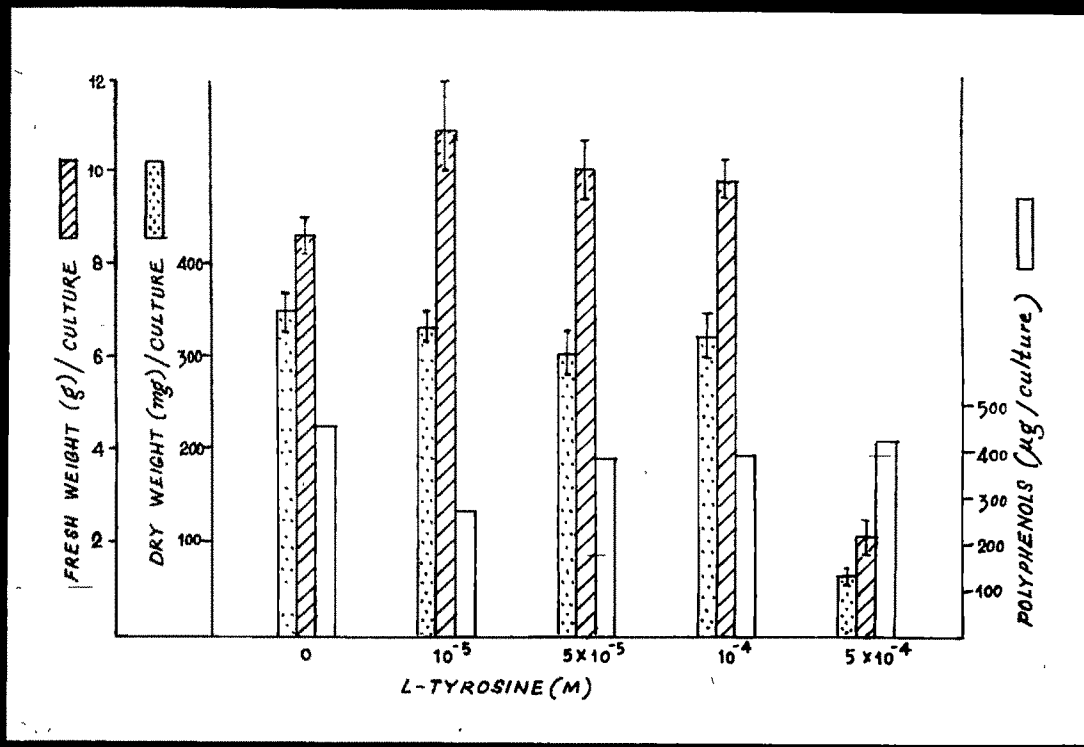
FRESH WEIGHT (g)/ CULTURE
DRY WEIGHT (mg)/CULTURE
POLYPHENOLS (µg/culture)
12
10
8
6
4
2
400
300
200
100
500
400
300
200
100
0
10⁻⁵
5X10⁻⁵
10⁻⁴
5 X10⁻⁴
L-TYROSINE (M)

Fig.18

to the control. In presence of low concentrations of tyrosine, there was some increase in fresh weight but the dry weight of the tissue decreased slightly. At the highest level of tyrosine tested, there was pronounced inhibition of growth without any significant effect on polyphenol production.

Experiment 4-8 : A Comparative Study on Growth and Production of Polyphenols in Datura Cells Grown on Solid and in Liquid Media

In order to compare the growth and total phenol production in tissues grown on solid or in liquid media, approximately 300±10 mg tissue by fresh weight was inoculated into Erlenmeyer flasks containing 40 ml of standard culture medium (Table 3, Chapter II). The solid medium was obtained by adding 0.9% agar. The volume of the medium and the size of the inoculum were kept constant in both the experiments.

The culture flasks containing liquid medium were agitated on a horizontal rotary shaker in light at constant temperature of 26±2°C. The flasks containing solid medium were also incubated in identical conditions of light and temperature.

Suitable number of replicates was harvested at 5 days interval upto 20 days for determining fresh and dry weights and for the estimation of total phenols.

The results presented in Table 14 and Fig. 19 and 20 showed that there was a general increase in total fresh and dry weights and total polyphenols with time in both the media tested. A prolonged lag was observed in fresh weight increase of the tissue in both the types of media; while the dry weights registered rapid raise from day 5 onwards in both the cases. Further, more pronounced increase in fresh and dry weights between day 10 and 15 was observed in cells grown in liquid medium than that registered by cells on solid medium extended even after 15 days; whereas the cells grown in liquid medium had almost attained stationary phase by then.

When the results on the production of total polyphenols were examined, polyphenol content was more in tissues grown in liquid medium (460 µg/culture) than in the case of the callus grown on solid medium (302 µg/culture). Total phenol production during lag phase of growth was negligible in tissues grown on both the solid and liquid media. Like dry weights, total phenol content rose rapidly after 5 days.

Table 14 : A Comparative Study on Growth and Production of Polyphenols in *Datura* Callus Cultures Grown on Solid and in Liquid Media*

Inoculum : 300±10 mg tissue by fresh weight in 40 ml of solid/liquid modified MS medium (Table 3, Chapter II).

Incubation: 20 days in light at 26±2°C.

Time (days)	Inoculum on solid medium 300±10 mg			Inoculum on liquid medium 300±10 mg		
	Fresh wt. (mg)	Dry wt. (mg)	Polyphenols (µg/culture)	Fresh wt. (mg)	Dry wt. (mg)	Polyphenols (µg/culture)
0	300±10 (5)	18 (3.0)	36 (0.4)	300±10 (5)	18 (3.0)	36 (0.4)
5	476 (8)	22 (2.2)	28 (0.6)	482 (7.4)	24 (2.8)	32 (0.8)
10	844 (25)	56 (8.0)	156 (1.2)	963 (12.6)	52 (10.3)	421 (3.6)
15	3124 (56)	172 (6.2)	296 (2.0)	8117 (38)	320 (8.0)	486 (2.8)
20	6028 (72)	256 (2.4)	302 (2.4)	8542 (42)	352 (8.1)	468 (3.2)

*Data represent average of six replicates.
Figures in the parenthesis represent standard error.

Fig. 19. Comparative study on growth of Datura cells grown on solid and in liquid media.

Inoculum size: 300±10 mg tissue in 40 ml of modified MS medium (Table 3, Chapter II).

Experimental details as given in Table 14.

Fig. 20. Comparative study on the changes in polyphenol content of Datura cells grown on solid or in liquid media.

Inoculum size: 300±10 mg tissue in 40 ml of modified MS medium (Table 3, Chapter II).

Experimental details as given in Table 14.

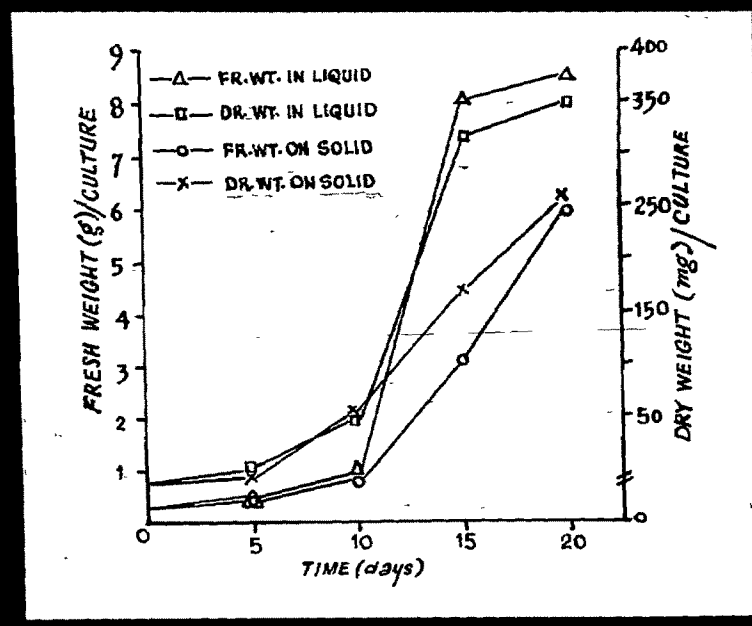
—Δ— FR.WT. IN LIQUID
—□— DR.WT. IN LIQUID
—o— FR.WT. ON SOLID
—x— DR.WT. ON SOLID
FRESH WEIGHT (g)/CULTURE
DRY WEIGHT (mg)/CULTURE
TIME (days)
0
1
2
3
4
5
6
7
8
9
50
150
250
350
400
5
10
15
20

Fig. 19

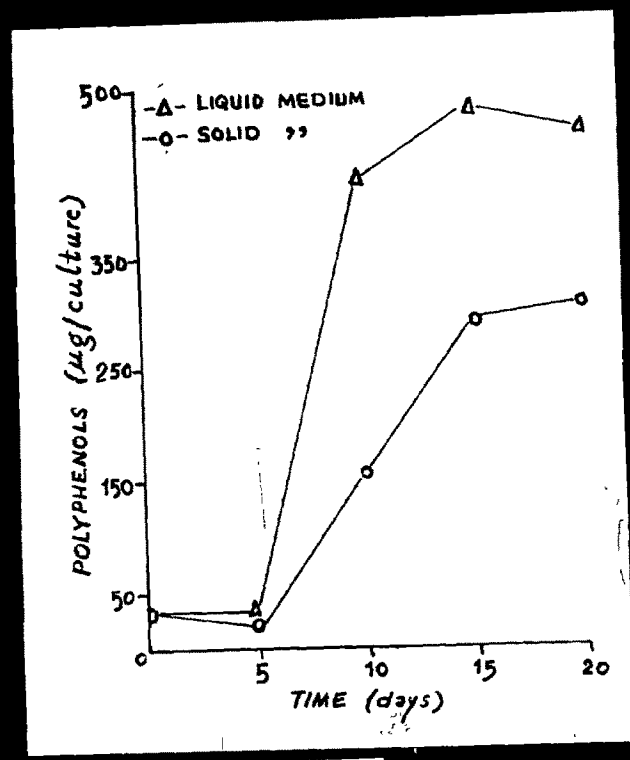
-Δ- LIQUID MEDIUM
-o- SOLID ,,
POLYPHENOLS (µg/culture)
TIME (days)
0
50
150
250
350
500
5
10
15
20

Fig. 20

In the case of tissues grown in liquid medium the raise was rather steep upto day 10 after which it slowed down. On the other hand, in tissues incubated on agar medium, there was a steady raise in polyphenol production upto day 15, before declining.

Experiment 4-9 : Effect of Inoculum Size on Growth and Polyphenol Production in Callus Cultures of Datura

Callus pieces of Datura each weighing 100 mg, 400 mg or 800 mg were separately inoculated in Erlenmeyer flasks containing 30 ml of culture medium (Table 3, Chapter II) solidified with 0.9% agar. The culture flasks were incubated in light at a constant temperature of 26±2°C. Suitable number of replicates was harvested at 5 days interval upto 20 days, for determining fresh weights and for the estimation of total phenols.

The results presented in Table 15 and Fig. 21 and 22 showed that there was a general increase in total fresh weight and polyphenol content with time in all the treatments. Pronounced lag phase, as measured by fresh weight, was observed when the inoculum size was low (100 mg). The lag appeared to be of least magnitude in the culture flasks

Table 15 : Effect of Inoculum Size on Growth and Polyphenol Production in Callus Cultures of Datura*

Inoculum : 100 mg, 400 mg or 800 mg tissue by fresh weight in 30 ml of modified MS medium (Table 3, Chapter II) supplemented with 2% sucrose, 2.0 mg/l 2,4-D and 0.4 mg/l kinetin.

Incubation: 30 days at 26±2°C in light.

Time (days)	INOCULUM : 100 mg		INOCULUM : 400 mg		INOCULUM : 800 mg	
	Fresh wt. (mg)	Polyphenols (µg/culture)	Fresh wt. (mg)	Polyphenols (µg/culture)	Fresh wt. (mg)	Polyphenols (µg/culture)
0	100	26	400	36	800	42
	(5)	(0.6)	(6)	(0.6)	(16)	(0.8)
5	156	18	550	30	850	45
	(6)	(0.4)	(8)	(0.4)	(14)	(0.6)
10	260	150	840	160	1052	185
	(16)	(1.4)	(8)	(0.8)	(24)	(1.5)
15	552	380	1083	275	1350	252
	(25)	(2.2)	(16)	(1.6)	(20)	(1.8)
20	2561	365	5800	260	6341	230
	(48)	(3.2)	(64)	(1.8)	(56)	(2.1)
30	4100	320	6078	250	6801	235
	(40)	(2.8)	(52)	(2.0)	(48)	(2.4)

*Data represent average of six replicates.
Figures in the parenthesis represent standard error.

Fig. 21. Effect of inoculum size on growth of *Datura* callus cultures.

Inoculum size: 100 mg, 400 mg or 800 mg tissue by fresh weight in 30 ml of modified MS medium (Table 3, Chapter II).

Experimental details as given in Table 15.

Fig. 22. Effect of inoculum size on polyphenol accumulation in *Datura* callus cultures.

Inoculum size: 100 mg, 400 mg or 800 mg tissue by fresh weight in 30 ml of modified MS medium.

Experimental details as given in Table 15.

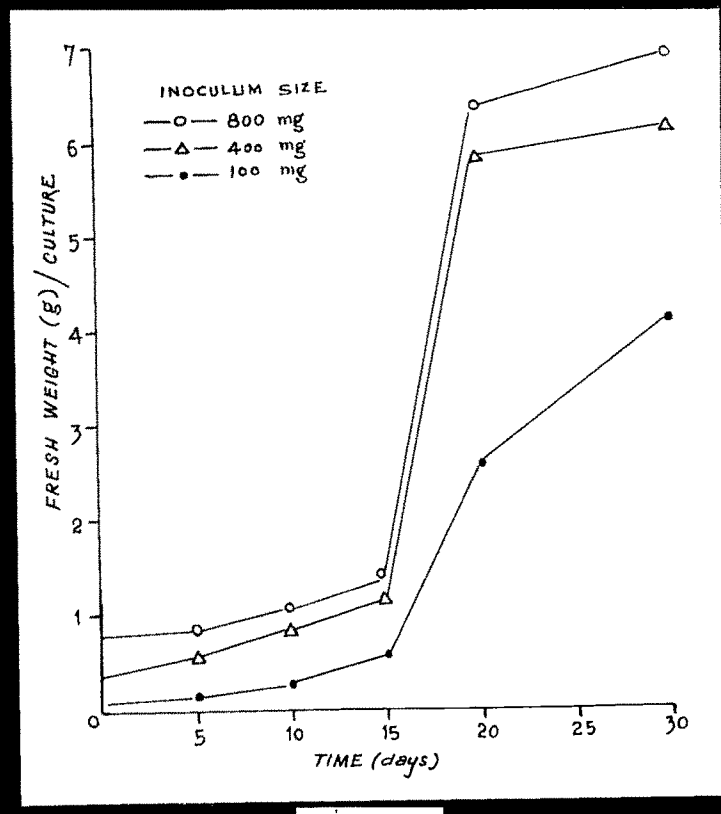
INOCULUM SIZE
800 mg
400 mg
100 mg
FRESH WEIGHT (g) / CULTURE
TIME (days)
0
1
2
3
4
5
6
7
5
10
15
20
25
30

Fig. 21

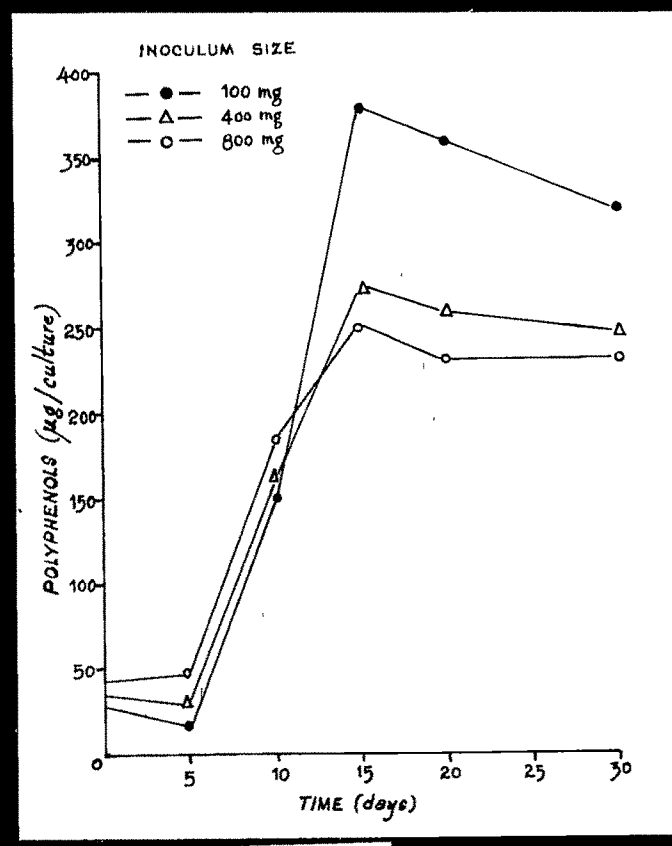
INOCULUM SIZE
100 mg
400 mg
800 mg
POLYPHENOLS (µg/culture)
TIME (days)
0
50
100
150
200
250
300
350
400
5
10
15
20
25
30

Fig. 22

containing 400 mg inoculum; at the inoculum size of 800 mg there was no corresponding reduction in lag. Further, the total growth attained was highest (30 fold increase in fresh weight) in cultures having low inoculum (100 mg) and least (8.5 fold increase in fresh weight) in cultures inoculated with high inoculum (800 mg). Furthermore, the fresh weights continued to increase in flasks containing low inoculum even after 20 days incubation, while growth in higher inoculum size attained more or less stationary phase on day 20.

Polyphenol production also increased with decrease in inoculum size; the highest increase (12 fold) being registered when inoculum size was small (100 mg). The maximum polyphenol production was recorded in pre-exponential phase of growth in all the inoculum sizes tested.

Experiment 4-10 : Effect of Light and Dark on Growth and Production of Total Phenols in Datura Callus Cultures Grown on Two Auxin Concentrations

Weighed amount of callus pieces (100±10 mg by fresh weight) were inoculated into 150 ml capacity culture flasks containing 30 ml of modified MS medium (Table 3, Chapter II)

supplemented with 10^{-5} or $5x10^{-5}$M 2,4-D in addition to 0.4 mg/l kinetin. The medium was solidified with 0.9% agar.

The flasks were incubated in an environmental chamber under controlled conditions where high intensity illumination (5,000 Lux) was given from cool white flourescent lamps. An another set of replicates was incubated in complete darkness. In both the cases the temperature and relative humidity were maintained at 25±0.5°C and 68% respectively.

A fixed number of replicate flasks was harvested at 5 days interval upto 20 days for the measurement of fresh and dry weights and for the estimation of total phenols accumulated in the tissues.

The results showing the effect of light and darkness on the growth and polyphenol accumulation are presented in Table 16 and illustrated in Fig. 23 and 24. In general the cultures exposed to light showed more polyphenol synthesis as compared to the cultures grown in darkness. Further, of the two auxin levels tested, $5x10^{-5}$M 2,4-D concentration was found to be inhibitory for polyphenol production in dark grown cultures. The stimulatory effect of light on polyphenol production was more pronounced and

Table 16 : Effect of Light and Dark on Growth and Polyphenol Production in *Datura* Callus Cultures Grown on Two Auxin Media*

Inoculum : 100±10 mg tissue by fresh weight in 30 ml of modified MS medium (Table 3, Chapter II) supplemented with 10^{-5} or $5x10^{-5}$M 2,4-D in addition to 0.4 mg/l kinetin.

Incubation: 20 days at 25±0.5°C and 68% humidity.

Time (days)	10^{-5}M 2,4-D						$5x10^{-5}$M 2,4-D					
	Dark			Light (5000 Lux)			Dark			Light (5000 Lux)		
	Fresh wt. (mg)	Dry wt. (mg)	Poly-phenols (µg/culture)	Fresh wt. (mg)	Dry wt. (mg)	Poly-phenols (µg/culture)	Fresh wt. (mg)	Dry wt. (mg)	Poly-phenols (µg/culture)	Fresh wt. (mg)	Dry wt. (mg)	Poly-phenols (µg/culture)
0	100 (3.22)	7 (0.42)	18 (0.4)	100 (3.22)	7 (0.42)	18 (0.4)	100 (3.22)	7 (0.42)	18 (0.4)	100 (3.22)	7 (0.42)	18 (0.4)
5	156 (3.51)	8 (0.21)	20 (0.5)	130 (4.24)	8 (0.62)	25 (2.4)	125 (2.98)	7 (0.42)	40 (0.8)	120 (3.38)	7 (0.38)	26 (0.3)
10	272 (4.04)	13 (0.48)	135 (1.3)	201 (2.46)	12 (0.32)	160 (2.3)	160 (2.72)	8 (0.86)	143 (2.2)	135 (2.48)	8 (0.62)	195 (2.3)
15	771 (2.95)	32 (0.28)	180 (2.5)	502 (5.28)	20 (0.12)	210 (2.5)	300 (2.26)	11 (1.25)	165 (2.4)	269 (4.26)	12 (0.21)	402 (2.5)
20	1556 (3.27)	58 (0.56)	201 (2.5)	1198 (8.92)	52 (0.52)	260 (1.5)	594 (6.72)	20 (0.82)	175 (2.0)	375 (6.24)	13 (0.52)	480 (2.4)

*Data represent average of six replicates.

Figures in the parenthesis represent standard error.

Fig. 23. Effect of light (5000 Lux) and dark on growth of Datura callus cultures grown on two auxin concentrations.

Inoculum size: 100±10 mg tissue in 30 ml of modified MS medium supplemented with 10^{-5} or $5x10^{-5}$M 2,4-D.

Experimental details as given in Table 16.

Fig. 24. Effect of light (5000 Lux) and dark on polyphenol production in Datura callus cultures grown on two auxin concentrations.

Inoculum size: 100±10 mg tissue in 30 ml of modified MS medium supplemented with 10^{-5} or $5x10^{-5}$M 2,4-D.

Experimental details as given in Table 16.

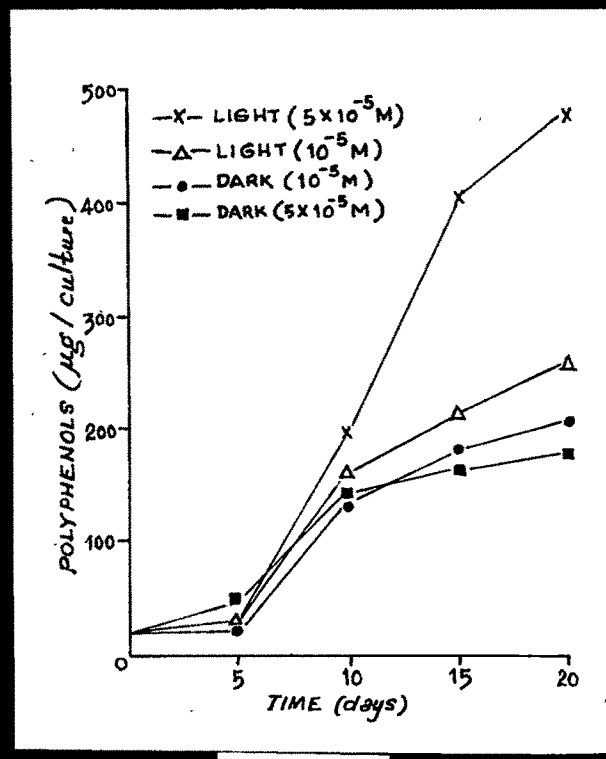
—X— LIGHT (5×10⁻⁵M)
—△— LIGHT (10⁻⁵M)
—●— DARK (10⁻⁵M)
—■— DARK (5×10⁻⁵M)
POLYPHENOLS (µg / culture)
500
400
300
200
100
0
5
10
15
20
TIME (days)

Fig. 23

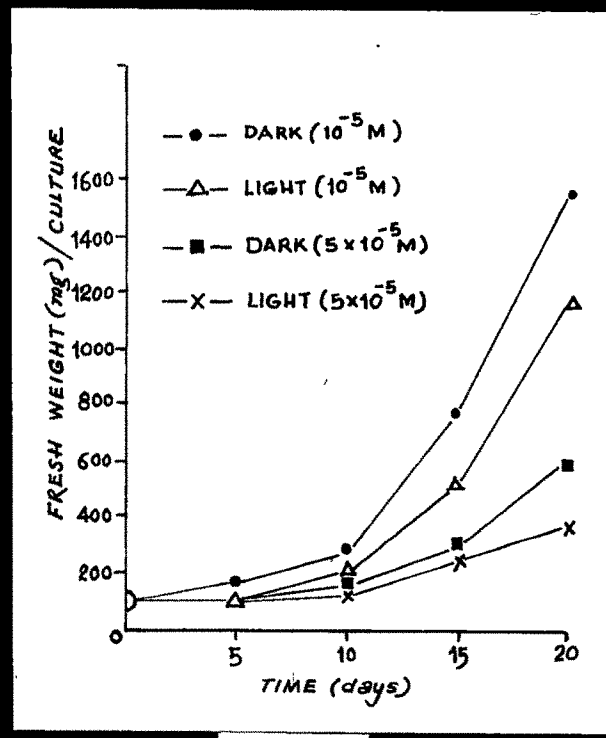
—●— DARK (10⁻⁵M)
—△— LIGHT (10⁻⁵M)
—■— DARK (5×10⁻⁵M)
—X— LIGHT (5×10⁻⁵M)
FRESH WEIGHT (mg)/CULTURE
1600
1400
1200
1000
800
600
400
200
0
5
10
15
20
TIME (days)

Fig. 24

marked at $5x10^{-5}$M 2,4-D than at lower auxin level. This showed that there is an interaction between light and auxin effects in that illumination reversed the inhibitory effects of high auxin levels on polyphenol production.

The results also revealed that high light intensity inhibited the growth of the tissue at both the auxin concentrations tested, the growth inhibition being more pronounced at high auxin ($5x10^{-5}$M) level present in the medium.

Experiment 4-11 : Effect of Gibberellic Acid on Growth and Polyphenol Production in Callus Cultures of Datura in Presence and in Absence of Light

Callus pieces weighing approximately 100±10 mg by fresh weight were inoculated into Erlenmeyer flasks containing 30 ml of defined agar medium (Table 3, Chapter II) supplemented with 10^{-6} or $3x10^{-6}$M gibberellic acid (GA_3) in addition to 2.0 mg/l 2,4-D and 0.4 mg/l kinetin.

The flasks were incubated in an environmental chamber having high light intensity (5000 Lux) provided by cool white flourescent lamps. Another set of flasks was kept in complete darkness under identical conditions of relative humidity (68%) and temperature (25±0.5°C).

A fixed number of replicate flasks was harvested at 5 days interval upto 20 days for the measurement of growth and for the determination of total phenol content in the tissues.

The results showing the effect of light and complete darkness, in presence and in absence of GA_3 are presented in Table 17 and illustrated in Fig. 25 and 26. In general the growth values of the cultures exposed to light was very much low both in absence and in presence of high concentrations of GA_3 ($3x10^{-6}M$). However, the growth of the tissues subjected to $10^{-6}M$ GA_3 concentration and exposed to light was considerably higher than in the control, suggesting the reversal of inhibitory effect of high intensity light on growth by low concentration of GA_3.

Light had promotory effect on polyphenol synthesis even in absence of GA_3. Further, GA_3 at low concentration ($10^{-6}M$) enhanced the total phenol production both in presence and in absence of light over control. However, higher concentrations of GA_3 ($3x10^{-6}M$) inhibited the accumulation of total polyphenol content both in presence and in absence of light, at all stages of the growth cycle.

Fig.25. Effect of gibberellic acid (GA_3) on growth of Datura callus cultures grown in presence and in absence of light.

Inoculum size: 100±10 mg tissue in 30 ml of modified MS medium supplemented with 10^{-6} or $3x10^{-6}$M gibberellic acid.

Experimental details as given in Table 17.

Fig. 26. Effect of gibberellic acid (GA_3) on polyphenol production in Datura callus cultures grown in presence and in absence of light.

Inoculum size: 100±10 mg tissue in 30 ml of modified MS medium supplemented with 10^{-6} or $3x10^{-6}$M GA_3.

Experimental details as given in Table 17.

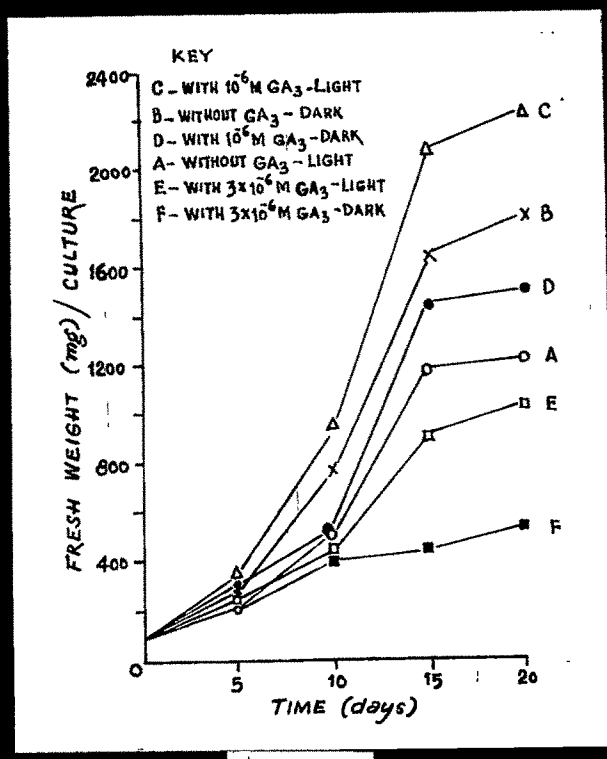
KEY
C - WITH 10^{-6}M GA_3 - LIGHT
B - WITHOUT GA_3 - DARK
D - WITH 10^{-6}M GA_3 - DARK
A - WITHOUT GA_3 - LIGHT
E - WITH 3×10^{-6}M GA_3 - LIGHT
F - WITH 3×10^{-6}M GA_3 - DARK
FRESH WEIGHT (mg) / CULTURE
2400
2000
1600
1200
800
400
0
5
10
15
20
TIME (days)
A
B
C
D
E
F

Fig. 25

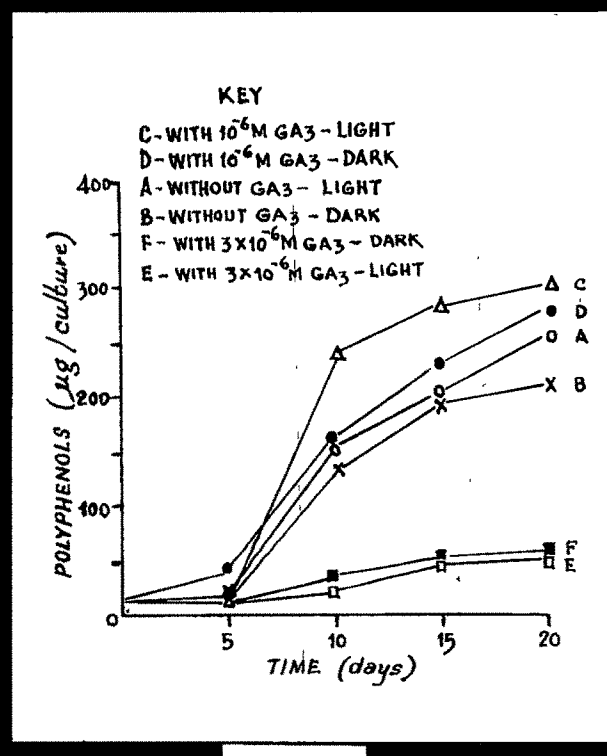
KEY
C - WITH 10^{-6}M GA3 - LIGHT
D - WITH 10^{-6}M GA3 - DARK
A - WITHOUT GA3 - LIGHT
B - WITHOUT GA3 - DARK
F - WITH 3×10^{-6}M GA3 - DARK
E - WITH 3×10^{-6}M GA_3 - LIGHT
POLYPHENOLS (µg / culture)
400
300
200
100
0
5
10
15
20
TIME (days)
A
B
C
D
E
F

Fig. 26

Table 17 : Effect of Gibberellic Acid on Growth and Polyphenol Production in Callus Cultures of Datura in presence and in Absence of Light*

Inoculum : 100± 10 mg tissue by fresh weight in 30 ml of medified MS medium (Table 3, Chapter II) supplemented with 0, 10^{-6} or $3x10^{-6}$M gibberellic acid (GA_3).

Incubation: 20 days at 25±0.5°C and 68% humidity.

Time (days)	Without GA_3						With 10^{-6}M GA_3						With $3x10^{-6}$M GA_3					
	Dark			Light			Dark			Light			Dark			Light		
	Fr.wt. (mg)	Dr.wt. (mg)	Polyphenols (ug/culture)	Fr.wt. (mg)	Dr.wt. (mg)	Polyphenols (ug/culture)	Fr.wt. (mg)	Dr.wt. (mg)	Polyphenols (ug/culture)	Fr.wt. (mg)	Dr.wt. (mg)	Polyphenols (ug/culture)	Fr.wt. (mg)	Dr.wt. (mg)	Polyphenols (ug/culture)	Fr.wt. (mg)	Dr.wt. (mg)	Polyphenols (ug/culture)
0	100	7	18	100	7	18	100	7	18	100	7	18	100	7	18	100	7	18
	(5.0)	(0.38)	(0.4)	(5.0)	(0.38)	(0.4)	(5.0)	(0.38)	(0.4)	(5.0)	(0.38)	(0.4)	(5.0)	(0.38)	(0.4)	(5.0)	(0.38)	(0.4)
5	272	13	20	202	12	20	302	12	40	356	15	22	213	9	20	240	10	12
	(3.24)	(0.24)	(0.3)	(5.8)	(0.46)	(0.6)	(6.0)	(0.50)	(0.8)	(5.2)	(0.68)	(0.6)	(3.1)	(0.16)	(0.4)	(4.2)	(0.12)	(0.6)
10	771	32	138	502	20	156	521	22	160	958	52	240	416	23	32	414	18	24
	(4.21)	(0.6)	(0.8)	(5.6)	(0.52)	(1.2)	(4.2)	(0.31)	(1.3)	(8.4)	(0.45)	(1.0)	(3.8)	(0.32)	(0.8)	(2.8)	(0.22)	(1.6)
15	1656	58	193	1198	52	208	1468	62	230	2086	94	286	422	24	55	906	36	46
	(2.56)	(0.24)	(1.1)	(4.8)	(0.12)	(1.8)	(3.2)	(0.28)	(2.0)	(10.2)	(0.56)	(1.8)	(4.6)	(0.25)	(1.1)	(8.2)	(0.52)	(1.4)
20	1808	65	210	1220	56	260	1503	68	278	2248	105	301	548	28	60	1034	39	48
	(3.28)	(0.58)	(2.2)	(6.7)	(0.26)	(2.0)	(2.2)	(0.14)	(2.2)	(6.9)	(0.32)	(2.0)	(6.4)	(0.34)	(2.2)	(6.2)	(0.84)	(2.8)

*Data represent average of six replicates.
Figures in the parenthesis represent standard error.

Experiment 4-12 : Morphogenesis and Polyphenol Synthesis in Callus Cultures of Cassia Grown on Defined Medium

The aim of the experiment described here was to induce morphogenesis and to find out correlation between the total polyphenol content and organogenic difference in the tissue cultures of Cassia.

The modified MS medium (Table 3, Chapter II) was supplemented with IAA (0, 0.05, 0.1 or 1.0 mg/l) or kinetin (0, 0.5 or 1.0 mg/l) and in combination as shown in the chart below:

		Kinetin (mg/l)		
		0.0	0.5	1.0
IAA (mg/l)	0.0			
	0.05	*		
	0.1	*		
	1.0			

Weighed amount (100±10 mg) of callus pieces were inoculated into 150 ml capacity Erlenmeyer flasks containing 30 ml of medium. The culture flasks were incubated in light at a constant temperature of 26±2°C.

On periodical examination, initiation of root primordia was observed in the callus pieces grown on 0.05 and 0.1 mg/l auxin alone (marked with * in the chart) for 15 to 20 days (Fig. 27 and 28). A fixed number of flasks of all the treatments, was harvested after incubation for 30 days for the determination of fresh and dry weights and for the estimation of polyphenol content in cultured tissues.

The results presented in Table 18 and Fig. 27 & 28 showed that in those callus pieces where there is differentiation of root primordia the polyphenol content was comparatively low. This seemed to indicate an inverse relationship between morphogenesis and polyphenol production.

Further, the results revealed that in absence of kinetin, IAA alone had no effect on the production of polyphenols. On the other hand, in absence of auxin there was marked increase in total polyphenol content in tissues grown on kinetin containing medium. Auxin and kinetin together, however, had a promotory influence on polyphenol production as compared to no-auxin-no-kinetin control.

The data presented in Tables 19 and 20 revealed that growth enhanced in presence of auxin or kinetin and also when both were supplied together. Growth increase was,

Table 18 : Effect of Auxin alone and in Combination with Kinetin on Polyphenol Production in Callus Cultures of *Cassia**

Inoculum : 300±20 mg by fresh weight of tissue in 30 ml of modified MS medium (Table 3, Chapter II) supplemented with 0.0, 0.05, 0.1 or 1.0 mg/l IAA and 0.0, 0.5 or 2.0 mg/l kinetin.

Incubation: 30 days at 26±2°C in light

IAA (mg/l)	Kinetin (mg/l) 0.0	0.5	2.0
0.0	883 (0.8)	3435 (2.6)	6320 (3.0)
0.05	1693 (1.4)	3236 (1.2)	4314 (1.6)
0.1	1815 (1.8)	3244 (2.0)	6787 (2.8)
1.0	1504 (1.2)	3804 (2.2)	5986 (2.4)

*Data represent average (µg/culture) of six replicates. Figures in the parenthesis represent standard error.

Fig. 27. Initiation of root primordia from Cassia anther callus grown on modified MS medium (Table 3, Chapter II) supplemented with 0.05 mg/l IAA and 2% sucrose.

Incubation : 30 days in light at 26±2°C.

Fig. 28. Initiation of root primordia from Cassia anther callus grown on modified MS medium (Table 3, Chapter II) supplemented with 0.1 mg/l IAA and 2% sucrose.

Incubation: 30 days in light at 26±2°C.

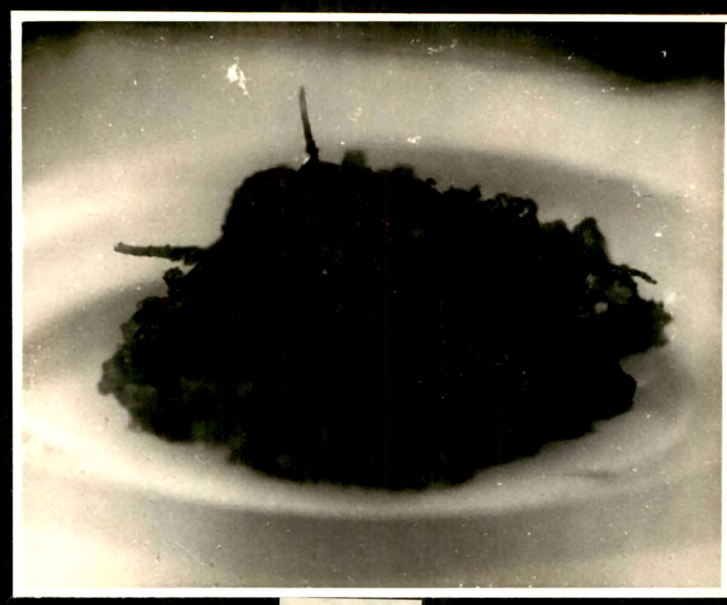

Fig. 27

Fig. 28

Table 19 : Effect of Auxin alone and in Combination with Kinetin on Growth (Fresh weight) of *Cassia* Callus Cultures*

Inoculum : 300±20 mg of tissue by fresh weight in 30 ml of modified MS medium (Table 3, Chapter II) supplemented with 0.0, 0.05, 0.1 or 1.0 mg/l IAA and 0.0, 0.5 or 2.0 mg/l Kinetin.

Incubation: 30 days at 26±2°C in light.

		Kinetin (mg/l)		
		0.0	0.5	2.0
IAA (mg/l)	0.0	879 (8.2)	2051 (18.2)	2866 (8.6)
	0.05	1993 (12.5)	2530 (20.5)	4209 (22.2)
	0.1	1806 (10.2)	2093 (16.4)	3878 (15.6)
	1.0	1583 (6.8)	3459 (12.3)	4518 (26.8)

*Data represent average (Fr.wt. mg/culture) of six replicates.
Figures in the parenthesis represent standard error.

Table 20 : Effect of Auxin alone and in Combination with Kinetin on Growth (Dry weight) of *Cassia* Callus Cultures*

Inoculum : 300±20 mg (Dry weight: 18 mg) of tissue in 30 ml of modified MS medium (Table 3, Chapter II) supplemented with 0.0, 0.05, 0.1 or 1.0 mg/l IAA and 0.0, 0.5 or 2.0 mg/l Kinetin.

Incubation: 30 days at 26±2°C in light.

		Kinetin (mg/l)		
		0.0	0.5	2.0
IAA (mg/l)	0.0	61 (1.24)	129 (2.42)	133 (1.62)
	0.05	85 (2.18)	102 (1.86)	267 (2.72)
	0.1	90 (1.68)	182 (3.21)	206 (3.86)
	1.0	98 (3.26)	174 (2.52)	251 (3.41)

*Data represent average (Dry wt. mg/culture) of six replicates.
Figures in the parenthesis represent standard error.

however, more pronounced in presence of kinetin alone than in presence of auxin alone. Synergestic effect on growth as measured by increase in fresh and dry weights was observed at high levels of auxin and kinetin tested.

DISCUSSION

It was clear from the experiment 4-1 that of the different sugars tested as energy source for growth and polyphenol production sucrose was the most efficient. Glucose and fructose when supplemented separately and together in equimolar mixture, supported nearly equal amount of growth; which, however, was poor as compared to the growth supported by sucrose. Further, equimolar mixture of glucose and fructose enhanced the production of polyphenols in the tissues thus proving next best to sucrose as carbohydrate source. The growth rate and total polyphenol production observed in the cells grown on medium containing maltose was not very significant and soluble starch was found to be the poorest source of energy. Thus superiority of sucrose for the production of total polyphenols and for supporting maximum growth in Datura cell suspensions was realised from Experiment 4-1.

After the superiority of sucrose as the best carbohydrate source for maximum growth and for higher

production of polyphenols was established, its effects at different concentrations were investigated (Experiment 4-2). When Datura cells grown under the influence of different concentrations of sucrose were analysed for polyphenol content, maximum accumulation was registered in cells grown on medium containing 4% sucrose. The results presented in Table 7 clearly suggested that the increased polyphenol production depended on the availability of sucrose as carbohydrate source during the growth cycle. The polyphenol content increased significantly during the pre-exponential phase of the growth cycle and the maximum accumulation of polyphenols was observed on day 15 after which it slowed down.

The effect of 2,4-D concentrations in presence of optimal level of kinetin ($2x10^{-6}$M) on growth and polyphenol accumulation in Datura callus cultures as shown in Fig. 12 and 13 (Experiment 4-3), suggested that the increasing auxin level delayed the initiation of polyphenol synthesis. At all concentrations tested, the polyphenol production was found to be virtually restricted to pre-exponential growth period. Accumulation of total phenols had apparently terminated at approximately same stage at all auxin concentrations and it was also observed that very high concentration ($5x10^{-5}$M) significantly reduced

the total phenol content. Furthermore, since the growth rate was markedly affected at different 2,4-D levels, it had also affected the polyphenol synthesis. In absence of 2,4-D the initial delay in polyphenol synthesis was not noticed.

In the following experiment (Experiment 4-4), kinetin was found to have no delaying influence on the initiation of polyphenol synthesis, except in its absence and at its low doses. The latter was perhaps, due to the presence of optimal level of 2,4-D in the medium, which had delayed the initiation of polyphenol synthesis as observed earlier (Experiment 4-3). The accumulation of total polyphenols was observed mainly in the pre-exponential phase, and like 2,4-D, the kinetin level optimal for growth also promoted maximum polyphenol production. Further, kinetin at different levels, had less pronounced influence on the accumulation of polyphenols in Datura cells in presence of optimal level of 2,4-D as compared to that of 2,4-D levels in presence of kinetin (Experiment 4-3).

Examination of suitable nitrogen source for polyphenol production and growth revealed that a balanced supply of potassium and ammonium nitrates was more effective than other organic and inorganic nitrogen source tested individually (Experiment 4-5).

L-phenylalanine and L-tyrosine, the aromatic amino acids formed in the shikimic acid pathway are known to function as precursors in the biosynthesis of phenylpropanoid compounds. These amino acids at different levels were, therefore, supplemented individually to the synthetic medium to examine their influence on polyphenol production in Datura cell suspensions. It was observed that L-phenylalanine at 10^{-5}M concentration considerably enhanced growth over control; while at higher doses the growth was supressed. At the highest concentration of phenylalanine, though growth was sharply reduced, the polyphenol synthesis was stimulated (Experiment 4-6). L-tyrosine, on the other hand, had no effect on polyphenol production; but it promoted growth by fresh weight at all concentrations tested except at the highest where there was marked retardation of growth (Experiment 4-7).

Comparison of the total polyphenol content in Datura cultures grown either on solid or in liquid medium revealed that maximum growth values (28 fold increase in fresh weight and 20 fold increase in dry weight) and higher amount of polyphenol accumulation were registered in liquid cultures (Experiment 4-8). This was perhaps

mainly because of the easy accessibility of the nutrients to all the free cells and possible increased aeration due to constant agitation of the liquid medium during culture period. Furthermore, like dry weights, the total phenol content also rose rapidly after an initial lag of 5 days, the raise being more steep in liquid cultures than in solid cultures.

The results presented in Experiment 4-9 clearly revealed that the growth and polyphenol production in Datura callus cultures were markedly influenced by the size of the inoculum. There was observed marked lag phase in the growth cycle when inoculum size was low (100±10 mg). The highest growth value (30 fold increase in fresh weight) was also registered in cultures containing low inoculum size and with the increase in inoculum size there was a corresponding decline in growth values (15 and 8 fold increase in fresh weight at 400 mg and 800 mg inoculum sizes respectively). A similar trend in polyphenol accumulation was also observed during the course of culture for 30 days at different inoculum loads. This seemed to imply the depletion of essential nutrients (or metabolites) for growth as well as polyphenol production.

The results obtained on the interaction of auxin and light of high intensity clearly showed that light stimulated polyphenol synthesis; further the stimulatory effect of light was much more marked at higher ($5x10^{-5}M$) 2,4-D level. The growth of the tissue, however, was inhibited by the high light intensity at both the auxin levels tested (Experiment 4-10).

On the other hand, in presence of light, GA_3 at $10^{-6}M$ concentration, promoted growth as well as total phenol production. Higher concentrations of GA_3 ($3x10^{-6}M$) were, however, found to be inhibitory on the growth of the tissue and also on polyphenol synthesis both in light and in dark (Experiment 4-11). This indicated that an optimal level of GA_3 further enhanced the stimulatory effect of light on polyphenol synthesis and also reversed the inhibitory effect of light on growth.

Studies on auxin/kinetin interaction in Cassia callus cultures (Experiment 4-12) suggested that a negative correlation exhisted between morphogenesis and polyphenol production. In those cultures where the initiation of root was observed (i.e. at 0.05 and 0.1 mg/l IAA alone) the polyphenol production was comparatively low. Further, in absence of kinetin, IAA alone had no significant effect

on the polyphenol production. However, in absence of IAA, kinetin at 0.5 and 2.0 mg/l levels stimulated the total polyphenol accumulation in Cassia callus cultures. Both IAA and kinetin, singly and in combination, promoted growth. At higher concentrations of both together, synergestic effect on growth was observed.