CHAPTER III

IN VITRO STUDIES ON GROWTH AND NUTRITION OF *DATURA* ANTHER CALLUS

IN VITRO STUDIES ON GROWTH AND NUTRITION OF DATURA ANTHER CALLUS

The experiments described in the present Chapter were aimed at successful establishment of tissue cultures from the anthers of *Datura metel*, a member of the family Solanaceae. As indicated in the Introduction (Chapter I), callus tissues derived from many plant parts could be grown in continuous culture on nutritive media which contain a utilizable carbohydrate, inorganic salts and other growth promoting substances. Using White's (1954) medium as basic composition, attempts were made to improve the growth of tissues by altering various cultural parameters. The cultural variables examined included: different sugars as energy sources, and different nitrates as nitrogen sources, and different levels of macro- and micro nutrient salts in the medium and different auxins. Relationship between the inoculum size and volume of the medium was also examined to investigate the growth limiting factor or factors. The experiments conducted to assess the influence of above variables on growth of established callus cultures are described in the present Chapter.

EXPERIMENT 1 : Initiation of Callus in excised Anthers of Datura

In order to determine the most suitable nutrient medium to initiate callus from the excised anthers of Datura and for its subsequent indefinite growth, segments of anthers at different stages of development, were placed on various solid media prepared as described in Materials and Methods 2,A. White's (1954) basal medium (Table 1) with the following supplements were tested for callus induction:-

a) 2,4-D (2.0 mg/l)
b) coconut milk (10 per cent, v/v)
c) casein hydrolysate (300 mg/l)
d) 2,4-D (2.0 mg/l) and coconut milk (10 per cent, v/v)
e) 2,4-D (2.0 mg/l) and casein hydrolysate (300 mg/l)
f) 2,4-D (2.0 mg/l), coconut milk (10 per cent, v/v) and casein hydrolysate (300 mg/l).

Anthers which had not reached the pollen grain stage failed to grow on any of the above nutrient media and eventually dried. Best growth was obtained on the medium ('f' above) containing 2,4-D, coconut milk and casein hydrolysate. The combined effects of 2,4-D and coconut milk were apparently synergistic because they had little effect on growth when supplied separately

Fig. 1A. Excised anther showing callus formation at the cut ends after two weeks of inoculation.

(An - Anther; Ca - callus)

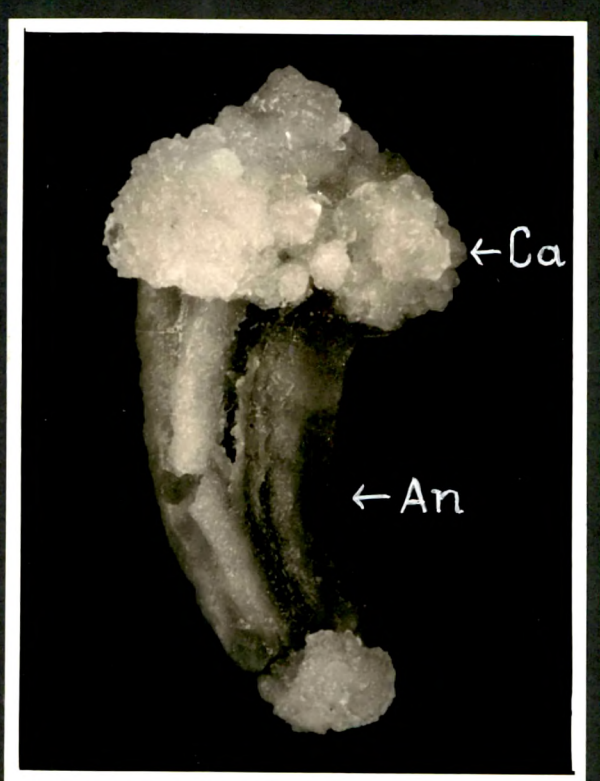


Fig. 1A

Fig. 1B. Anther explant showing callus after four weeks of inoculation.

(An - Anther; Ca - callus).

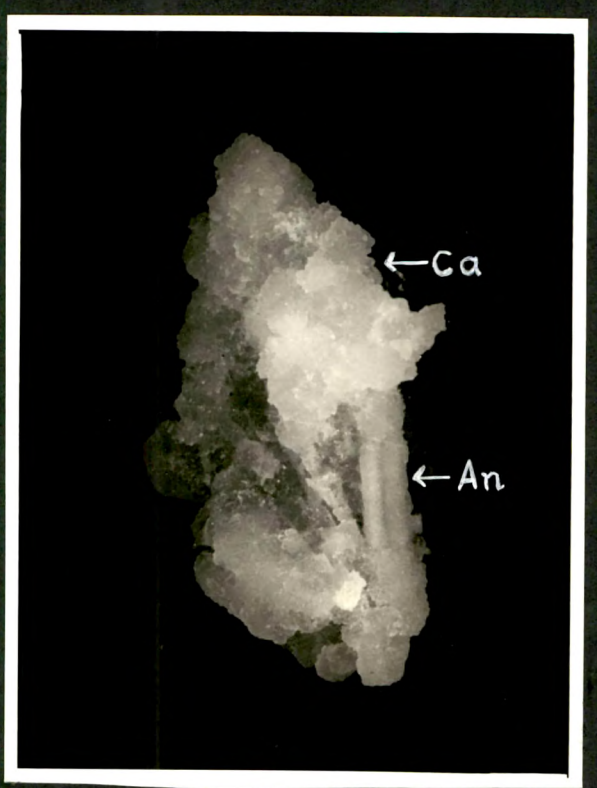


Fig. 1B

Fig. 1C. Transverse section of part of the anther showing callus formation from the connective.

(Co - connective; CA - Callus) 10x5x.

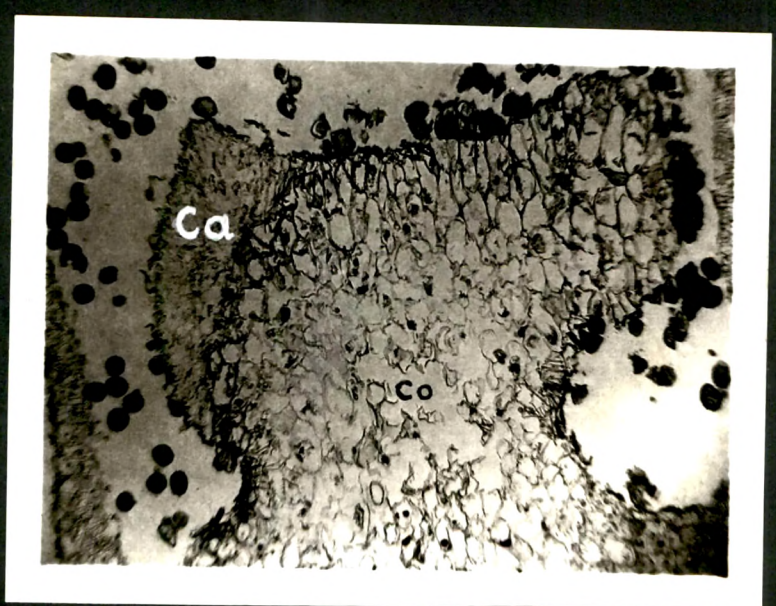


Fig. 1C

Fig. 1D. Cells of *Datura* anther callus.

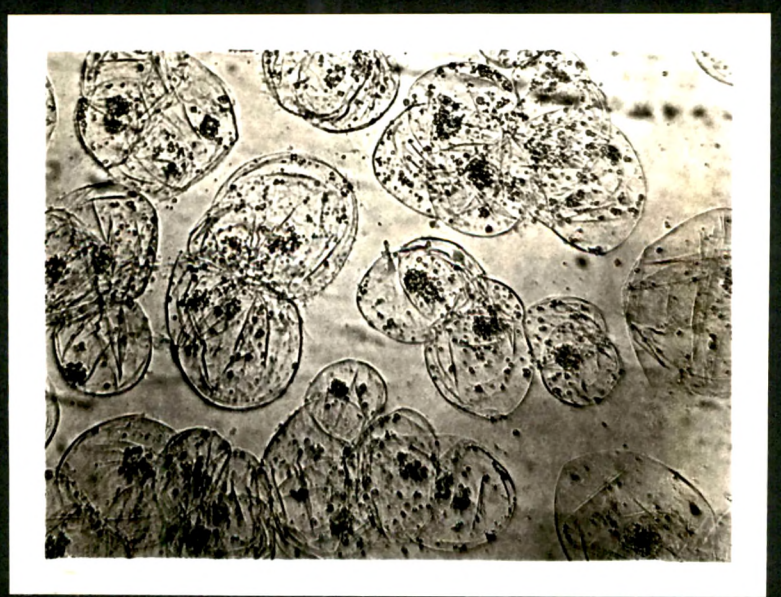


Fig. 1D

(media 'a' and 'b'). When added together, they produced profuse proliferation at the cut ends of the anthers (Fig. 1 A). Within four weeks of incubation, the original explant was completely over-grown by a mass of callus tissue (Fig. 1 B). Incorporation of casein hydrolysate further enhanced growth of callus tissue; and was therefore added routinely to the medium.

To determine the anatomical origin of the callus formed on the anther segments, serial sections were cut at different stages of callus development (Material and Methods, 9). Microscopic observations revealed that the callus initiated from the connective (Fig. 1 C) to begin with and later on the anther wall cells also started proliferating. Squash preparations of fresh callus tissue showed losely arranged, parenchymatous mass of irregularly shaped cells. The latter were uninucleate and contained starch grains (Fig. 1 D).

EXPERIMENT 2 : Effect of different Auxins on Callus Growth

Experiments were then conducted to find out the specific auxin requirement for rapid and continuous callus growth. The callus tissues developed and maintained on 2,4-D medium were kept on auxin free medium for a period of 2 weeks before inoculation of the experiment to test

the effect of different auxins on growth.

Tissue pieces each weighing 100±10 mg (fresh weight) were next inoculated in 100 ml Erlenmeyer flasks containing 25 ml of the experimental medium (Table 1) supplemented with:-

a) IAA (1.0 mg/l), coconut milk (10 per cent) and casein hydrolysate (300 mg/l)
b) NAA (1.0 mg/l), coconut milk (10 per cent) and casein hydrolysate (300 mg/l)
c) 2,4-D (2.0 mg/l), coconut milk (10 per cent) and casein hydrolysate (300 mg/l).

The measurements of different growth parameters after incubation of the cultures for a period of four weeks in light at 26±2°C are presented in Table 5 and Figure 2. Clearly, at the concentrations tested, 2,4-D promoted highest growth as measured by fresh weight, dry weight and total cell number of the tissue. IAA and NAA caused almost similar enhancement in dry weight and total cell number of the tissue. 2,4-D on the other hand, showed clear edge over the other two auxins.

Further experiments were conducted to find out the optimal concentration of 2,4-D for callus growth. The auxin concentrations tested were: 0.0, 0.5, 1.0, 2.0 and

Table 5 : Effect of different Auxins on Growth of *Datura metel* Linn. Callus tissue*

Inoculum : 100±10 mg of tissues (Dry weight: 5.8 mg and Total Cell No. 0,04 x 10^6) in 25 ml of White's medium supplemented with coconut milk (10%), casein hydrolysate (300 mg/l) and either IAA (1.0 mg/l), NAA (1.0 mg/l) or 2,4-D (2.0 mg/l).

Incubation : 4 weeks in light at 26±2°C.

Auxins	Fresh Wt (mg)	Dry Wt (mg)	Total Cell No. (x 10^6)
IAA (1.0 mg/l)	3330 (50)	121 (4.5)	1.35 (0.03)
NAA (1.0 mg/l)	3410 (38)	128 (4.0)	1.42 (0.04)
2,4-D (2.0 mg/l)	4220 (49)	170 (4.5)	1.80 (0.04)

* Figures in the parentheses represent standard error.

Table 6 : Effect of 2,4-Dichlorophenoxyacetic acid on Growth of *Datura* Callus cultures*

Inoculum : 100±10 mg of tissue (Dry weight : 5.9 and Total Cell No. 0.04 x 10^6) in 25 ml of White's medium supplemented with coconut milk (10%) casein hydrolysate (300 mg/l) and different concentrations of 2,4-D.

Incubation : 4 weeks in light at 26±2°C.

2,4-D Conc (mg/l)	Fresh Wt (mg)	Dry Wt (mg)	Total Cell No. (x 10^6)
0.0	1812 (75)	96 (2.5)	0.9 (0.04)
0.5	2734 (75)	115 (4.0)	1.02 (0.05)
1.0	4028 (62)	158 (6.2)	1.63 (0.03)
2.0	4620 (80)	191 (5.0)	1.90 (0.04)
4.0	4060 (87)	161 (4.5)	1.56 (0.04)

* Figures in the parentheses represent standard error.

Fig. 2. Effect of different Auxins on Growth of *Datura* Callus cultures.

100 mg of tissues in 25 ml of White's medium supplemented with 1.0 mg/l IAA, 1.0 mg/l NAA or 2.0 mg/l 2,4-D.

Other experimental details as given in Table 5.

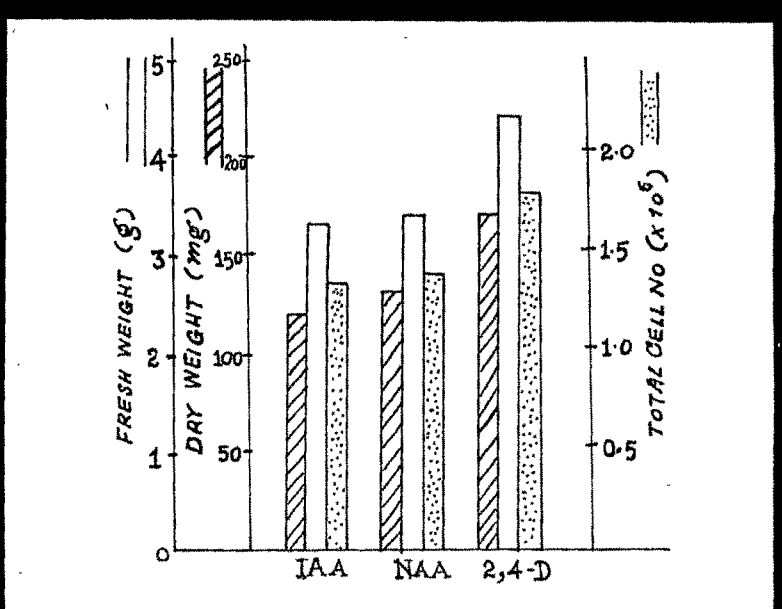

FRESH WEIGHT (g)
DRY WEIGHT (mg)
TOTAL CELL NO (x 10⁶)
0
1
2
3
4
5
50
100
150
200
250
0.5
1.0
1.5
2.0
IAA
NAA
2,4-D

Fig. 2

Fig. 3. Effect of 2,4-Dichlorophenoxyacetic acid on Growth of *Datura* Callus cultures.

100 mg of tissue in 25 ml of White's medium supplemented with 0.0, 0.5, 1.0, 2.0 or 4.0 mg/l 2,4-D.

Other experimental details as given in Table 6.

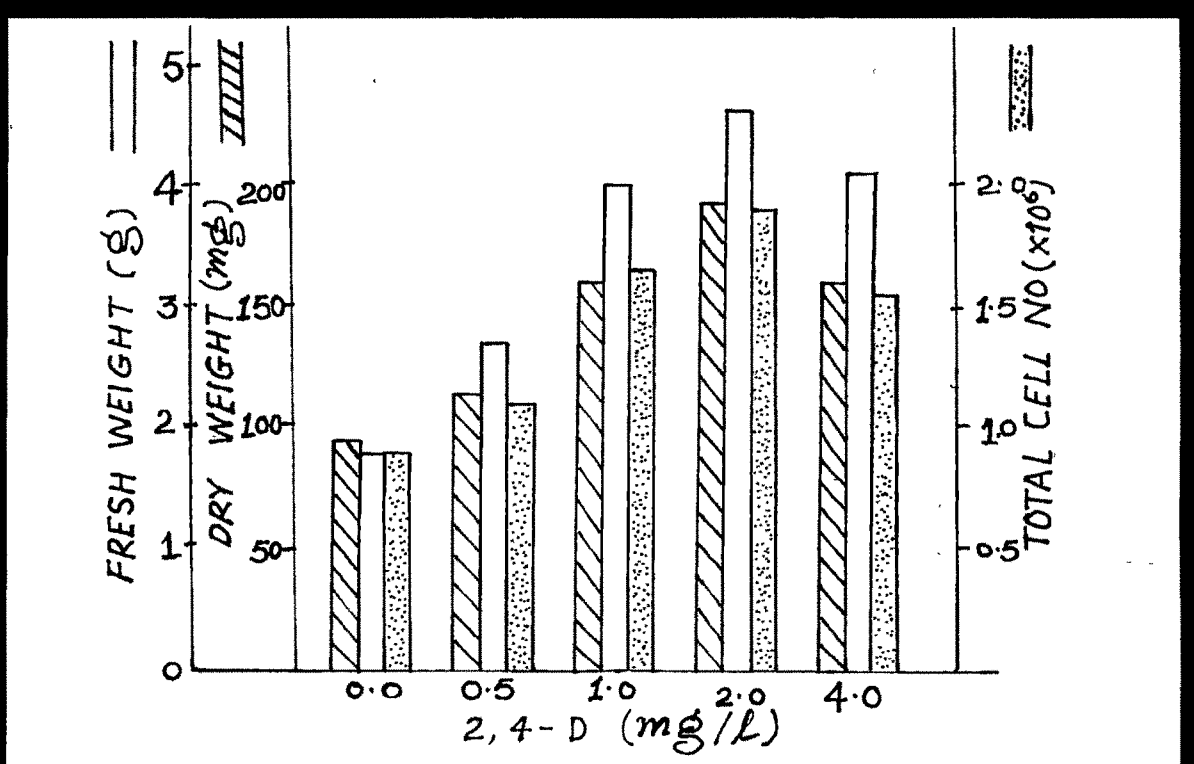

FRESH WEIGHT (g)
DRY WEIGHT (mg)
TOTAL CELL NO (x10⁶)
0
1
2
3
4
5
50
100
150
200
0.5
1.0
1.5
2.0
0.0
0.5
1.0
2.0
4.0
2, 4 - D (mg/l)

Fig. 3

4.0 mg/l. The results obtained after incubation for four weeks are presented in Table 6 and Figure 3.

As could be seen, 2.0 mg/l 2,4-D gave the maximum growth as measured by three different parameters. Growth increased with the increase in the 2,4-D concentration upto 2.0 mg/l and with further increase in the auxin concentration growth declined.

EXPERIMENT 3 : Effect of Macroelements of different culture Media on Growth of Datura Anther Callus

To determine the relative merits of macroelements as present in White's (1954), Heller's (1953) and Murashige and Skoog's (1962) (Tables 1,2 and 3) culture media for the growth of Datura anther callus, weighed pieces (100±10 mg) of callus tissues were inoculated in culture flasks containing the respective media. While the levels of macroelement salts were altered the microelements, vitamins and other supplements were incorporated as given in White's medium (Table 1). Ten replicates were made of each of the treatments. After incubation for four weeks in light, at 26±2°C the culture vessels were harvested to determine the fresh weight, dry weight and total cell number (Materials and

Table 7 : Effect of Macroelements of different Culture Media on Growth of *Datura* Callus tissues*

Inoculum : 100±10 mg of tissue (Dry weight: 5.9 mg and Total Cell No. 0.04 x 10^6) in 25 ml of:-

A. White's Macroelements,

B. Heller's Macroelements,

C. Murashige and Skoog's Macroelements and White's Microelement salts, coconut milk (10%) 2,4-D (2.0 mg/l) and 300 mg/l casein hydrolysate.

Incubation : 4 weeks in light at 26±2°C.

Medium	Fresh Wt (mg)	Dry Wt (mg)	Total Cell No. (x 10^6)
A	4060	160	1.65
	(33)	(1.8)	(0.03)
B	5530	220	2.30
	(86)	(3.8)	(0.02)
C	3048	120	1.30
	(93)	(3.8)	(0.04)

* Figures in the parentheses represent standard error.

Fig. 4. Effect of Macroelement salts of different Culture Media on Growth of Datura Callus tissue.

100 mg of tissue in 25 ml of Macroelement salts according to White's, Heller's or Murashige and Skoog's.

Other experimental details as given in Table 7.

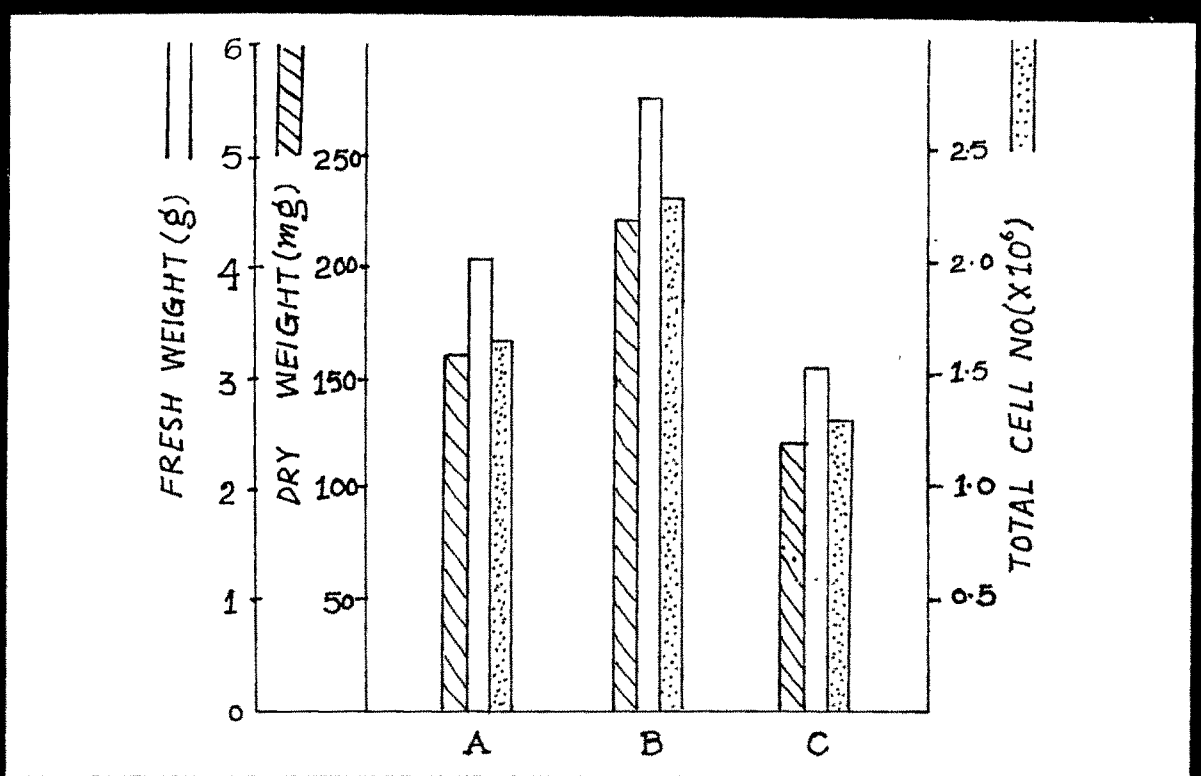

FRESH WEIGHT (g)
DRY WEIGHT (mg)
TOTAL CELL NO (X10⁶)
0
1
2
3
4
5
6
50
100
150
200
250
0·5
1·0
1·5
2·0
2·5
A
B
C

Fig. 4

Methods, 5A,B and C).

The results presented in Table 7 and Figure 4 showed that the growth of callus tissue was markedly more on the medium containing inorganic salts according to Heller than those containing salts according to White or Murashige and Skoog. The final dry weight and total cell number of tissues incubated on media containing Heller's macroelement salts were almost double those of tissues grown on Murashige and Skoog's macroelement salts. The increment in all the three growth parameters examined was appreciably more in Heller's as compared to White's on which the tissue cultures were initiated and maintained so far.

The tissues were, therefore, transferred henceforth onto the medium containing Heller's macroelement salts. The microelement salts and vitamins, however, were added according to White's. The above combination of salts supplemented with coconut milk 10 per cent, 2,4-D (2.0 mg/l) and casein hydrolysate (300 mg/l) was used for subsequent studies in the present investigation. The said complete medium is given in Table 4 and is henceforth referred to as Datura medium.

EXPERIMENT 4 : Growth curve of Datura

After several sub-cultures on Datura medium (Table 4) when the callus became white and looked healthy, weighed

(100±10 mg) pieces of callus were placed in Erlenmeyer flasks containing 25 ml of Datura medium (Table 4). The cultures were incubated in light at 26±2°C. Eight replicates were sacrificed every week to determine the dry weight and total cell number of the tissues. The results are presented in Table 8 and Figures 5A and 5B.

The graph of dry weight and total cell number plotted against time showed the usual type of sigmoid growth curve (Fig. 5A). Data for dry weight and total cell number when ploted on semi-log basis (Fig. 5B) showed that after an initial lag phase, growth was rapid during the second and third week and at the end of four weeks, increase in growth had slowed down. During the course of incubation for 6 weeks, there was an overall 38 fold increase in dry weight and 57 fold increase in total cell number. The rate of growth was, however, highest between 2 and 3 weeks, when there was recroded over 5 fold increase in dry weight and nearly 8 fold increase in total cell number. The latter corresponded to almost 3 cell generations in 7 days, indicating the mean cell generation time of little over 2 days during this period of maximum growth.

Table 8 : Progress of Growth in *Datura* Anther Callus cultures*

Inoculum : 100± mg of tissue (Dry weight: 60 mg and Total Cell No.0.04 x 10^6) in 25 ml of *Datura* medium

Incubation : 6 weeks in light at 26±2°C.

Time (Weeks)	Dry Wt (mg)	Total Cell No. (x 10^6)
1	8.4 (0.5)	0.07 (0.005)
2	36 (1.5)	0.26 (0.015)
3	192 (4.5)	2.00 (0.050)
4	218 (4.0)	2.20 (0.055)
5	228 (2.2)	2.29 (0.045)
6	230 (3.5)	2.28 (0.022)

* Figures in the parentheses represent standard error.

Fig. 5A and B. Progress of Growth in *Datura* Anther Callus culture.

100 mg of tissue in 25 ml of Datura medium (Table 4).

Other experimental details as given in Table 8.

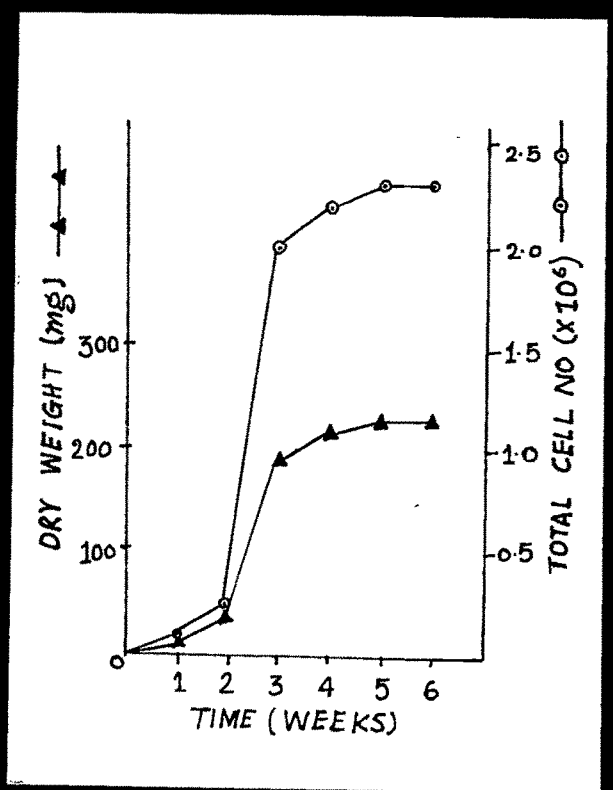

DRY WEIGHT (mg)
300
200
100
2·5
2·0
1·5
1·0
0·5
TOTAL CELL NO (X10⁶)
1 2 3 4 5 6
TIME (WEEKS)

Fig. 5A

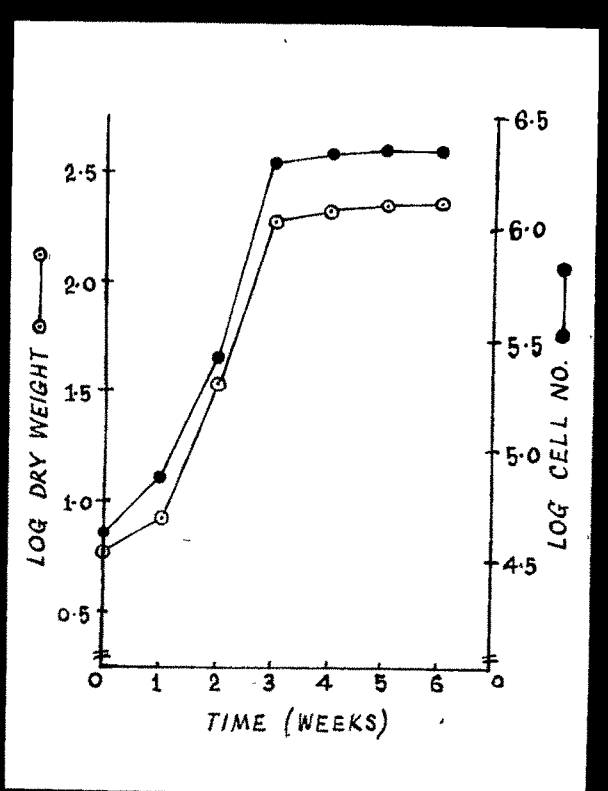

LOG DRY WEIGHT
2·5
2·0
1·5
1·0
0·5
6·5
6·0
5·5
5·0
4·5
LOG CELL NO.
0 1 2 3 4 5 6
TIME (WEEKS)

Fig. 5B

EXPERIMENT 5 : Effect of various Sugars and Different levels of Sucrose on Growth of Datura Callus culture

Experiments were conducted to find out the suitable carbohydrate requirement for continuous and rapid growth of Datura callus.

A. Effect of Sugars:

Tissue pieces of Datura callus weighing 100±10 mg each were inoculated separately in Erlenmeyer flasks containing 25 ml of:

Datura medium minus sugar (Medium A)
" " with 2% sucrose (Medium B)
" " " 2% glucose (Medium C)
" " " 2% fructose (Medium D)
" " " 2% equimolar mixture of glucose and fructose (Medium E)
" " " 2% maltose (Medium F)
" " " 2% soluble starch (Medium G).

Prior to the inoculation of the experiment, the callus tissues were grown on sugar free medium for a period of two weeks to minimise carry-over effect.

The growth responses of callus cultures incubated for four weeks in light at 26±2°C to various sugars are presented in Table 9 and Figure 6. Clearly starch was

the poorest source of energy for the growth of callus tissues; while sucrose (Medium B) supported the maximum growth (about 37 fold increase in dry weight and 57 fold increase in total cell number). Growth attained when glucose (Medium C) and fructose (Medium D) were supplied separately was almost similar to that when equimolar mixture of glucose and fructose was added together in the medium E (about 24 fold increase in dry weight and 37 fold increase in total cell number). Growth of the callus grown on maltose containing medium (Medium F) was not significantly less than that on media with either glucose (C), or fructose (D) or both (E).

B. Effect of Sucrose:

Experiment was next conducted to find out the optimal concentration of sucrose for the growth of Datura callus tissue. Callus tissues kept on media devoid of sucrose for two weeks were used to inoculate this experiment.

Erlenmeyer flasks containing 25 ml of Datura medium minus sucrose (Medium A) or supplemented with 0.5 (Medium B), 1.0 (Medium C), 1.5 (Medium D), 2.0 (Medium E) or 4.0 (Medium F) per cent sucrose were separately inoculated with callus tissues weighing 100±10 mg each.

Table 9 : Effect of Sugars on Growth of *Datura* Callus cultures*

Inoculum : 100±10 mg tissue (Dry weight: 6.0 mg and Total Cell No. 0.04 x 10^6) in 25 ml of Datura medium supplemented with:-

A. Datura medium minus sugar
B. Datura medium with 2% sucrose
C. Datura medium with 2% glucose
D. Datura medium with 2% fructose
E. Datura medium with 1% glucose + 1% fructose
F. Datura medium with 2% maltose
G. Datura medium with 2% starch

Incubation : 4 weeks in light at 26±2°C.

Medium	Dry Wt. (mg)	Total Cell No. (x 10^6)
A	30 (2.0)	0.3 (0.012)
B	215 (5.0)	2.3 (0.025)
C	147 (1.2)	1.5 (0.041)
D	146 (4.0)	1.5 (0.025)
E	150 (2.2)	1.5 (0.045)
F	140 (1.2)	1.4 (0.025)
G	116 (1.2)	1.2 (0.025)

* Figures in the parenthesis represent standard error.

Table 10 : Effect of Sucrose on Growth of *Datura* Callus cultures*

Inoculum : 100±10 mg of tissue (Dry weight: 6.0 mg and Total Cell No. 0.04 x 10^6) in 25 ml of:

A. Datura medium minus sugar
B. Datura medium with 0.5% sucrose
C. Datura medium with 1.0% sucrose
D. Datura medium with 1.5% sucrose
E. Datura medium with 2.0% sucrose
F. Datura medium with 4.0% sucrose

Incubation : 4 weeks in light at 26±2°C

Medium	Dry Wt (mg)	Total Cell No. (x 10^6)
A	30 (2.0)	0.3 (0.021)
B	69 (2.2)	0.8 (0.032)
C	142 (4.0)	1.5 (0.025)
D	183 (2.0)	1.9 (0.051)
E	230 (5.0)	2.3 (0.045)
F	202 (3.0)	1.7 (0.041)

* Figures in the parentheses represent standard error.

Table 11 : Effect of Sucrose on Per Cell Dry Weight* of *Datura* Callus cultures

Inoculum : 100±10 mg of tissue (Dry weight: 6.0 mg and Total Cell No.0.04 x 10^6) in 25 ml of:

A. Datura medium minus sugar
B. Datura medium with 0.5% sucrose
C. Datura medium with 1.0% sucrose
D. Datura medium with 1.5% sucrose
E. Datura medium with 2.0% sucrose
F. Datura medium with 4.0% sucrose

Incubation : 4 weeks in light at 26±2°C.

Medium	Dry Wt
A	0.100
B	0.086
C	0.094
D	0.096
E	0.100
F	0.119

* Values are multiple of 10^{-6}

Fig. 6. Effect of sugars on Growth of Datura Callus cultures.

100 mg of tissue inoculated in 25 ml of Datura medium either without sucrose (A) or with 2% sucrose (B), glucose (C), fructose (D), glucose-fructose (1:1) (E), maltose (F) or soluble starch (G).

Other experimental details as given in Table 9.

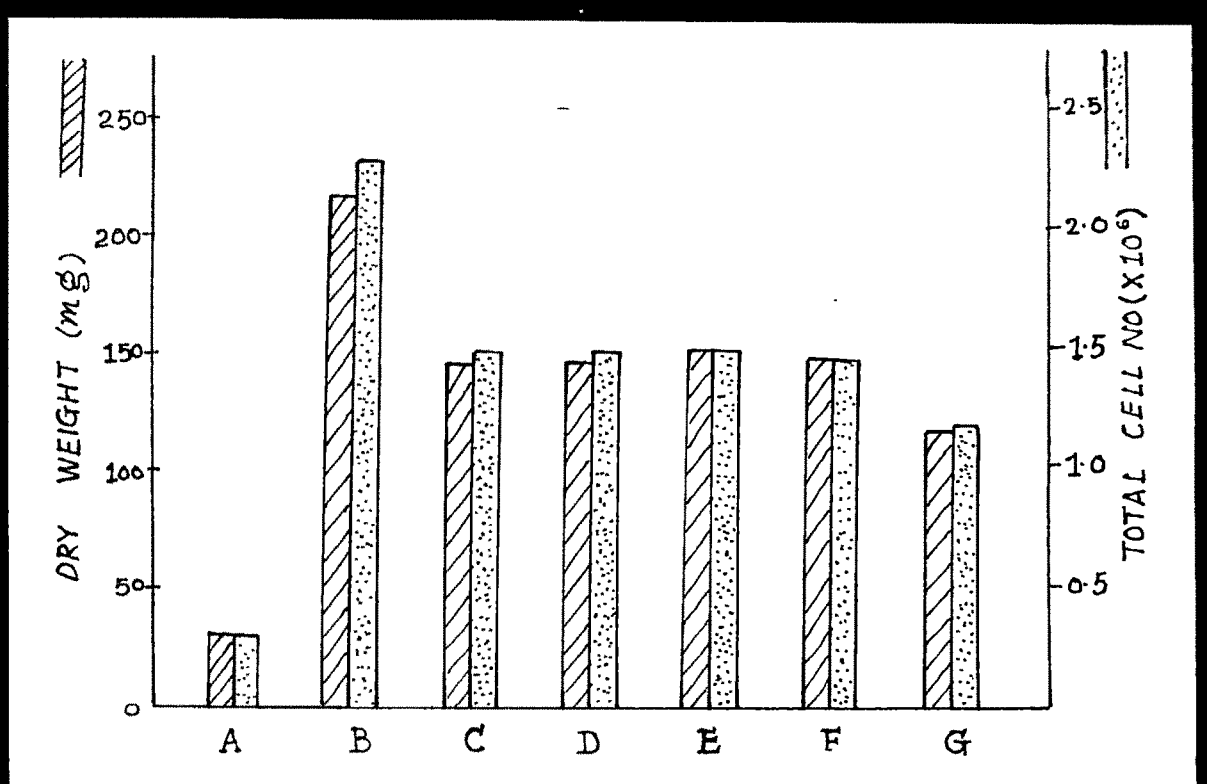

DRY WEIGHT (mg)
250
200
150
100
50
0
2·5
2·0
1·5
1·0
0·5
TOTAL CELL NO (X10⁶)
A
B
C
D
E
F
G

Fig. 6

The determination of growth of the tissues were made after incubation for 4 weeks in light at 26±2°C.

Data presented in Table 10 and Figure 7 showed that absence of sucrose from the nutritive medium resulted in very little growth. Growth as measured by increase in dry weight and total cell number increased with increasing levels of sucrose from 0.0 to 2.0 per cent. Further increase in sucrose level (4 per cent) reduced the dry weight as well as total cell number.

When dry weight was calculated on per cell basis (Table 11) it was noticed that there was progressive rise in cell dry weight with increase in sucrose (from 0.5 to 4.0 per cent) concentration.

EXPERIMENT 6 : Effect of Kinetin alone and in combination with Auxin on Growth of Datura Callus culture

A. Effect of Kinetin on Growth of Datura Callus cultures:

The callus cultures of Datura were transferred to auxin free medium for two weeks before inoculation of this experiment. Weighed amount (100±10 mg) of callus tissues were then inoculated in Erlenmeyer flasks

Fig. 7. Effect of sucrose concentration on Growth of *Datura* Callus culture.

100 mg of tissues inoculated in 25 ml of Datura medium containing different levels of sucrose.

Other experimental details as given in Table 10.

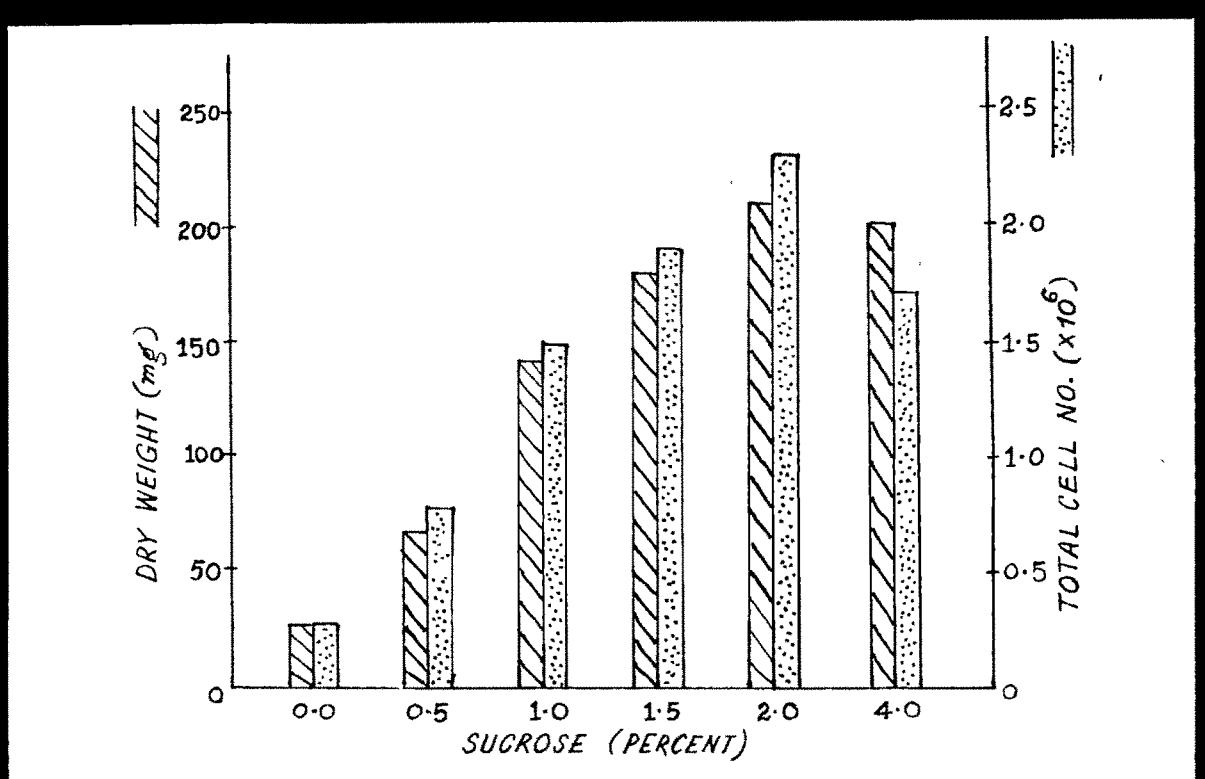

DRY WEIGHT (mg)
250
200
150
100
50
0
2·5
2·0
1·5
1·0
0·5
0
TOTAL CELL NO. (x10⁶)
0·0
0·5
1·0
1·5
2·0
4·0
SUCROSE (PERCENT)

Fig. 7

Table 12 : Effect of Kinetin (in absence of auxin) on Growth of *Datura* Callus cultures*

Inoculum : 100±10 mg of tissue (Dry weight: 6.0 mg and Total Cell No.0.04 x 10^6) in 25 ml of Datura medium (without 2,4-D) supplemented with 0.005, 0.05, 0.5 or 5.0 mg/l Kinetin.

Incubation : 4 weeks in light at 26±2°C.

Kinetin (mg/l)	Fresh Wt (mg)	Dry Wt (mg)	Total Cell No. (x 10^6)
0.0	1620 (25)	64 (2.5)	0.6 (0.012)
0.005	1990 (27)	92 (3.0)	0.8 (0.025)
0.05	3260 (45)	141 (4.0)	1.3 (0.050)
0.5	4192 (49)	158 (5.2)	1.7 (0.050)
5.0	2700 (38)	115 (2.5)	1.1 (0.025)

* Figures in the parentheses represent standard error.

Fig. 8. Effect of kinetin (in absence of auxin) on Growth of Datura Callus cultures.

100 mg of tissue in 25 ml of Datura medium (Table 4).

Other experimental details as given in Table 12.

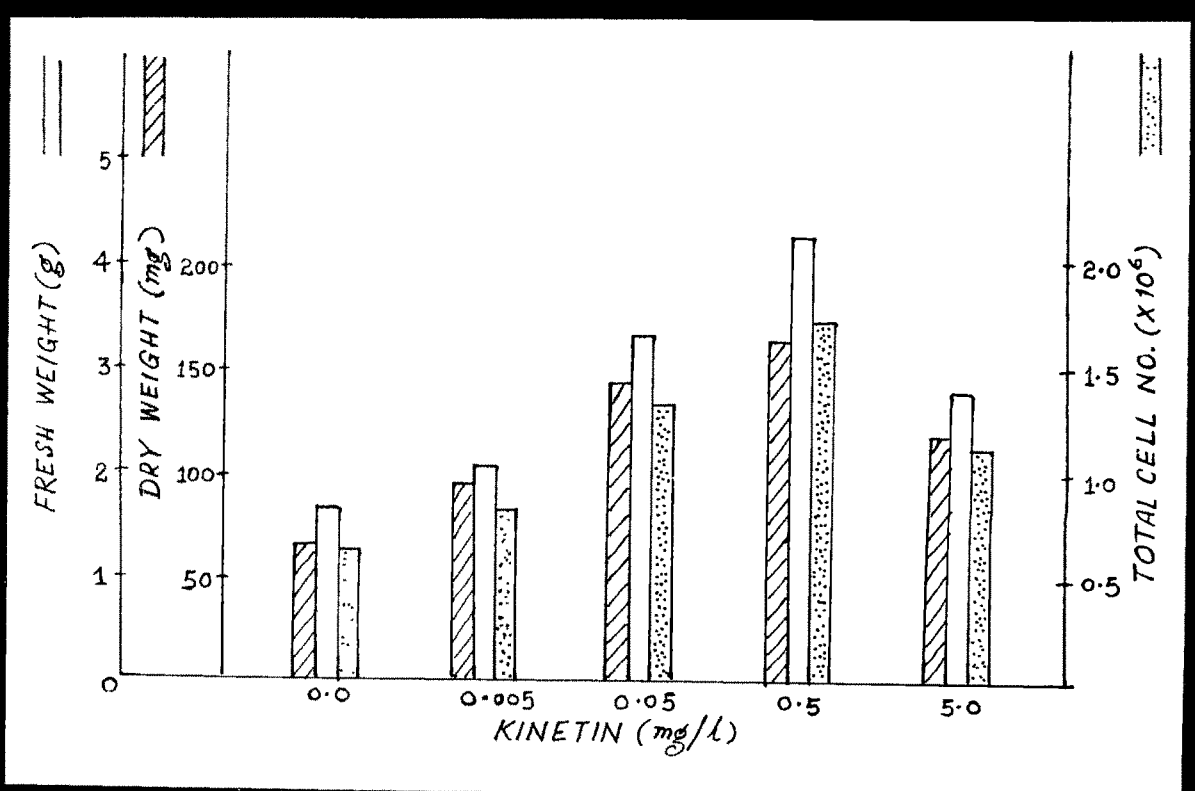

FRESH WEIGHT (g)
DRY WEIGHT (mg)
TOTAL CELL NO. (X 10⁶)
0
1
2
3
4
5
50
100
150
200
0·5
1·0
1·5
2·0
0·0
0·005
0·05
0·5
5·0
KINETIN (mg/l)

Fig. 8

containing 25 ml of Datura medium (without auxin) (Table 4) supplemented with 0.0, 0.005, 0.05, 0.5 or 5.0 mg per litre kinetin. Ten replicates were made for each treatment, and the final growth measurements were made after incubation for 4 weeks in light at 26±2°C.

The results presented in Table 11 and Figure 8 showed that in absence of auxin fresh weight, dry weight and total cell number increased with increasing kinetin concentration upto 0.5 mg/l. Maximum growth (fresh weight increase 42 fold, dry weight increase 26 fold and total cell number increase 42 fold) was registered in 0.5 mg/l containing medium. Higher levels of kinetin (5.0 mg/l) showed reduction in all the three parameters of growth.

B. Effect of Kinetin on Growth of Datura Callus cultures in presence of Standard Auxin:

Experiment was next conducted to find out the effect of kinetin on growth of Datura callus in the presence of standard auxin (2.0 mg/l 2,4-D) in the medium. Weighed amount (100±10 mg) of callus tissues were inoculated in Erlenmeyer flasks containing 25 ml of Datura medium (Table 4) supplemented with 0.0, 0.005, 0.05, 0.5 or 5.0 mg/l kinetin. The cultures were incubated for 4 weeks in light at 26±2°C.

Table 13 : Effect of Kinetin on Growth of *Datura* Callus cultures*

Inoculum : 100±10 mg of tissue (Dry weight: 6.0 mg and Total Cell No. 0.04 x 10^6) in 25 ml of Datura medium (containing 2.0 mg/l 2,4-D) supplemented with 0.005, 0.05, 0.5 or 5.0 mg/l Kinetin.

Incubation : 4 weeks in light at 26±2°C.

Kinetin (mg/l)	Fresh Wt (mg)	Dry Wt (mg)	Total Cell No. (x 10^6)
0.0	5160	213	2.20
	(40)	(3.7)	(0.025)
0.005	5220	217	2.20
	(37)	(5.0)	(0.041)
0.05	5284	223	2.25
	(32)	(3.7)	(0.052)
0.5	5490	236	2.45
	(22)	(3.5)	(0.012)
5.0	5064	202	2.10
	(41)	(4.2)	(0.025)

* Figures in the parentheses represent standard error.

Fig. 9. Effect of kinetin (in presence of 2.0 mg/l 2,4-D) on Growth of Datura Callus cultures.

100 mg of tissue in 25 ml of Datura medium (Table 4).

Other experimental details as given in Table 13.

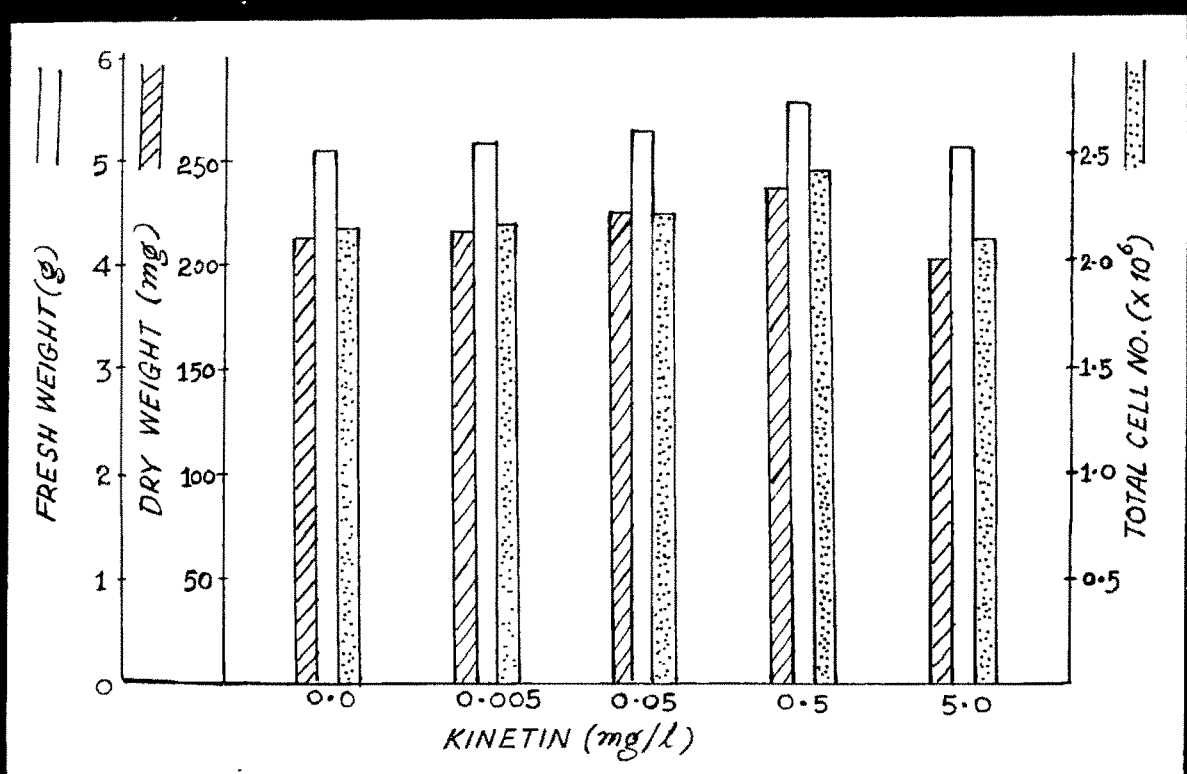

FRESH WEIGHT (g)
DRY WEIGHT (mg)
TOTAL CELL NO. (X 10⁶)
0
1
2
3
4
5
6
50
100
150
200
250
0.5
1.0
1.5
2.0
2.5
0·0
0·005
0·05
0·5
5·0
KINETIN (mg/l)

Fig. 9

Growth measurements presented in Table 13 and Figure 9, showed that in presence of standard auxin (2.0 mg/l 2,4-D), there was general enhancement of growth with increasing level of kinetin upto 0.5 mg/l in the medium. 0.5 mg/l kinetin in the medium containing 2.0 mg/l 2,4-D supported highest callus growth (61 fold increase in total cell number and 39 fold increase in dry weight). With further increase in kinetin concentration growth declined.

EXPERIMENT 7 : Relationship between Inoculum size and Volume of the medium as measured in terms of Growth of Datura Callus cultures

To examine how far the final growth attained was limited by the availability of nutrition supplied in a given volume of medium, the relationship between the inoculum size and volume of the medium was studied.

A. Effect of Inoculum size :

Tissue pieces of Datura, each weighing either 50, 100, 150 or 200±10 mg were separately inoculated in Erlenmeyer flasks containing 25 ml of Datura medium (Table 4). The culture flasks were incubated in light at 26±2°C for a period of 4 weeks. Ten replicates were kept for each treatment. The results are presented in Table 14 and Figure 10.

Table 14 : Effect of Inoculum size on Growth of *Datura* Callus cultures*

Inoculum : 50 mg (Dry weight : 3.0 mg and Total Cell No. 0.02 x 10^6), 100 mg (Dry weight: 6.0 mg and Total Cell No. 0.04 x 10^6), 150 mg (Dry weight: 9.0 mg and Total Cell No. 0.06 x 10^6), or 200 mg (Dry weight: 12.0 mg and Total Cell No. 0.08 x 10^6) of tissues in 25 ml of Datura medium.

Incubation : 4 weeks in light at 26±2°C.

Inoculum (mg)	Fresh Wt (mg)	Dry Wt (mg)	Total Cell No. (x 10^6)
50	4740	198	1.94
	(55)	(3.0)	(0.036)
100	5320	212	2.20
	(44)	(4.0)	(0.044)
150	5430	216	2.25
	(34)	(2.2)	(0.036)
200	5611	220	2.20
	(40)	(3.1)	(0.044)

* Figures in the parentheses represent standard error.

Fig. 10. Effect of Inoculum size on Growth of *Datura* Callus culture.

50, 100, 150 or 200 mg of tissue in 25 ml of Datura medium.

Other experimental details as given in Table 14.

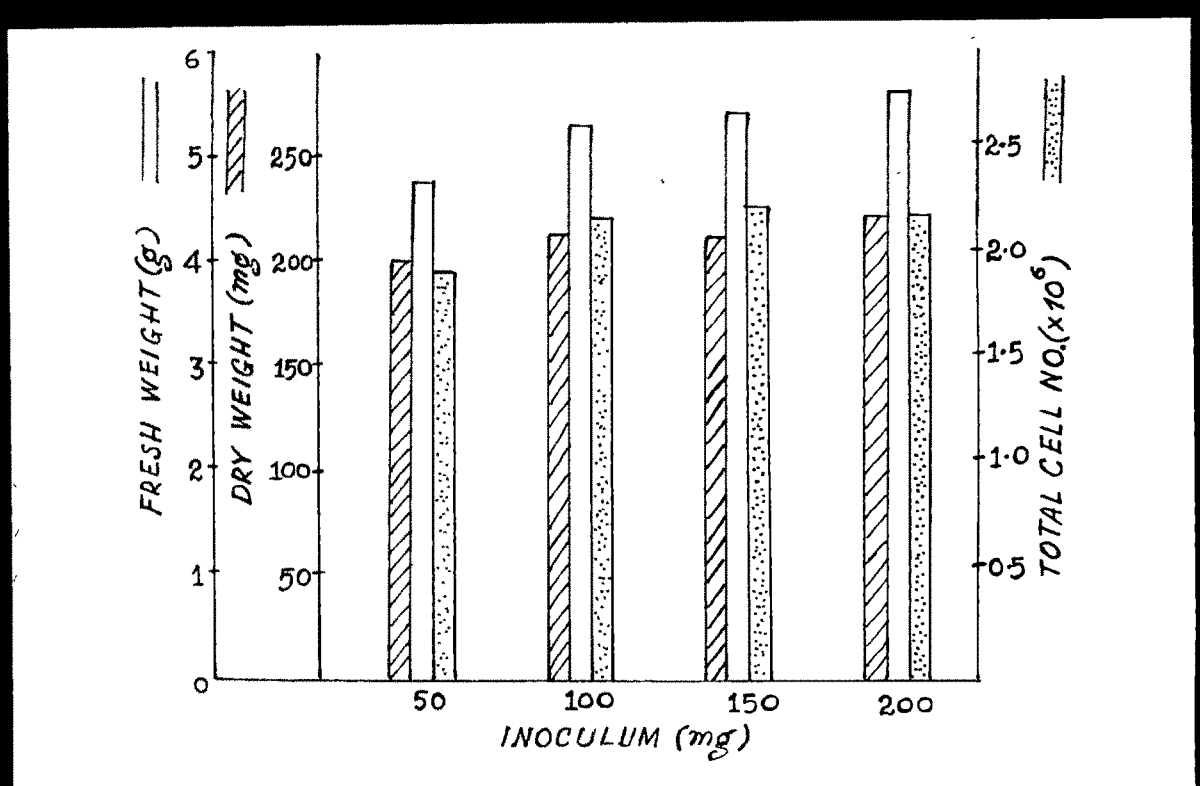

FRESH WEIGHT (g)
DRY WEIGHT (mg)
TOTAL CELL NO. (x10⁶)
0
1
2
3
4
5
6
50
100
150
200
250
0·5
1·0
1·5
2·0
2·5
50
100
150
200
INOCULUM (mg)

Fig. 10

Maximum total cell number production (95 fold) and highest increase in fresh weight (95 fold) and dry weight (66 fold) occured when the inoculum was low (50 mg) and with the increase in inoculum size there was corresponding decline in fresh weight (28 fold), dry weight (18 fold) and total cell number (27 fold). The number of cell generations decreased from 6.5 in 50 mg to 4.75 in 200 mg of inoculum. Following the percentage increase in dry weight and total cell number at different inoculum sizes, it was confirmed that 50 mg inoculum gave the highest values and the percentage increase in dry weight as well as total cell number progressively decreased with increasing inoculum size. Clearly the final growth attained was inversely related to the initial inoculum size in a fixed volume of the medium.

B. Effect of Volume of Culture medium :

To determine the effect of different volumes of culture media, uniform (100±10 mg) inocula of tissue were separately transferred in Erlenmeyer flasks containing 20, 40 or 60 ml of the culture medium. After incubation for 4 weeks in light at 26±2°C, growth measurements were made.

Table 15 : Effect of Volume of Culture Medium on Growth of *Datura* Callus tissue*

Inoculum : 100±10 mg of tissues (Dry weight: 6.0 mg and Total Cell No. 0.04 x 10^6) in 20, 40 or 60 ml of Datura medium.

Incubation : 4 weeks in light at 26±2°C.

Medium volume (ml)	Fresh Wt (mg)	Dry Wt (mg)	Total Cell No. (x 10^6)
20	4785	198	1.95
	(61)	(3.5)	(0.038)
40	6099	239	2.40
	(58)	(4.5)	(0.054)
60	8466	348	3.45
	(75)	(7.0)	(0.062)

* Figures in the parentheses represent standard error.

Fig. 11. Effect of Volume of Culture Medium on Growth of Datura Callus culture.

100 mg of tissue in 20, 40 or 60 ml of Datura medium.

Other experimental details as given in Table 15.

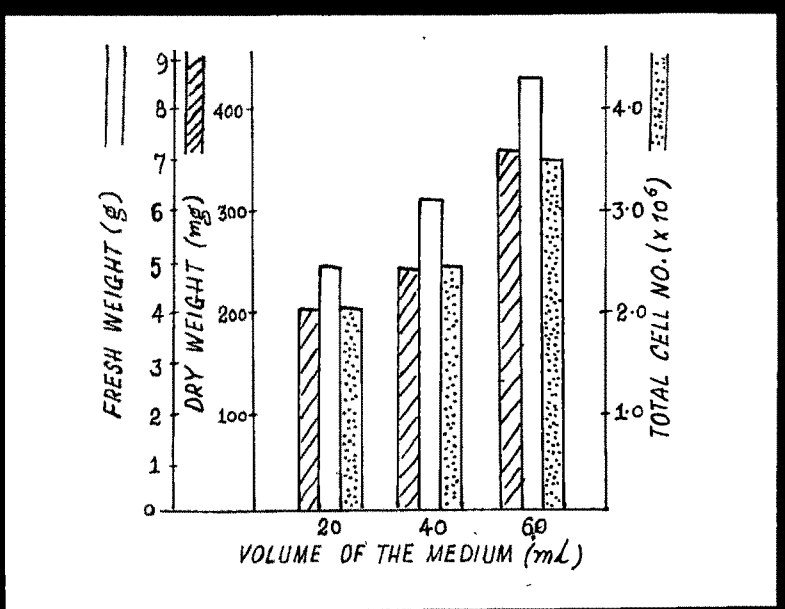

FRESH WEIGHT (g)
DRY WEIGHT (mg)
TOTAL CELL NO. (x 10⁶)
VOLUME OF THE MEDIUM (mL)
0
1
2
3
4
5
6
7
8
9
100
200
300
400
1·0
2·0
3·0
4·0
20
40
60

Fig. 11

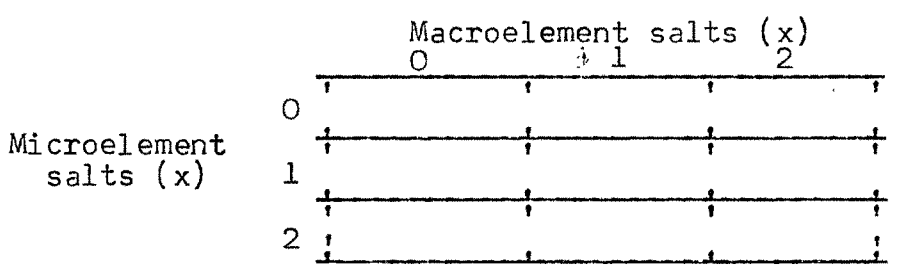
The results presented in Table 15 and Figure 11 showed that there was a general rise in fresh weight, dry weight and total cell number in all the treatments. Maximum total cell number (87 fold) and highest increase in dry weight (58 fold) were registered in the largest volume of the medium (60 ml) and with decrease in volume, there was corresponding decline in all the growth parameters. The number of cell generations increased from 5.5 in 20 ml of medium to around 6.4 in 60 ml volume of the medium. Percentage increase in dry weight and total cell number showed, however, gradual decline with increasing volume of the medium.

These results demonstrated that a given volume of the medium supported growth of a fixed cell population (about 210±10 mg dry weight and 2.0×10^6 total cell number) and the final yield of cells or tissues was dependent upon the volume of the medium rather than upon the initial inoculum size.

EXPERIMENT 8 : Effect of Inorganic Nutrition on Growth of Datura Callus cultures

To examine if the limiting factor of growth was exhaustion of some essential nutrient, the effect of macro-and microelement salts supplied at increasing levels in the culture medium on growth of callus tissue was studied.

A. Effect of Macro - and Microelement Salts:



Measured amounts of tissues, 100±10 mg by fresh weight, were transferred to Erlenmeyer flasks containing 25 ml of nutrient media with various combinations of macro - and microelement salts of Datura medium (Table 4) as shown above. Preincubation of the tissues for two weeks in media devoid of macro- or microelement salts was done to minimise any carryover effect. After incubation in light at 26±2°C for a period of 4 weeks, final growth measurements were made as described in Materials Methods, 5A,B and C.

Table 16 : Effect of the levels of Macroelement and Microelement Salts on Growth of *Datura* Callus cultures*

Inoculum : 100±10 mg tissue (Dry weight: 6.0 mg and Total Cell No. 0.04 x 10^6) in 25 ml of Datura medium with xo, xi or x2 macro- and microelement salts.

Incubation : 4 weeks in light at 26±2°C.

Microelement salt solution		Macroelement salt solution X0	Macroelement salt solution X1	Macroelement salt solution X2
X0	Fresh Wt (mg)	1489 (45)	3085 (45)	3309 (70)
	Dry Wt (mg)	66 (2.0)	126 (3.0)	135 (4.5)
	Total Cell No. (x 10^6)	0.6 (0.012)	1.2 (0.040)	1.3 (0.045)
X1	Fresh Wt (mg)	1647 (53)	5366 (64)	6432 (77)
	Dry Wt (mg)	71 (3.0)	229 (5.0)	258 (2.7)
	Total Cell No. (x 10^6)	0.7 (0.040)	2.1 (0.045)	2.7 (0.052)
X2	Fresh Wt (mg)	1985 (39)	5169 (58)	6094 (81)
	Dry Wt (mg)	79 (2.5)	220 (4.8)	242 (4.5)
	Total Cell No. (x 10^6)	0.8 (0.045)	2.0 (0.051)	2.6 (0.053)

* Figures in the parentheses represent standard error.

The data presented in Table 16 showed that in absence of macroelements, increasing levels of microelements did not cause appreciable enhancement of growth. In absence of microelement salts, on the other hand, there was marked enhancement of growth particularly as measured by increase in fresh and dry weight - with increasing level of macroelements in the medium.

In presence of standard level of microelements, doubling the level of macroelements resulted in marked increase in growth of callus tissue as measured by all the three parameters (dry weight 52 fold and total cell number 65 fold). In presence of standard macroelements in the medium, doubling the level of microelements caused slight reduction in growth. Similar decrease in growth was observed in presence of double level of macroelements.

B. Effect of the Total concentration of the Macroelement salts and the Individual ions of these salts on Datura Callus Growth:

Experiment was next conducted to find out the effect of total concentration of the macroelement salts and their individual ions on growth of Datura callus. The callus tissues growing on Datura medium (Table 4)

were transferred onto the nutrient medium devoid of macroelement salts to minimise carryover effect. After two weeks incubation on above medium, the tissues were used as inocula for this experiment.

Tissue pieces, weighing 100±10 mg each by fresh weight, were transferred separately to Erlenmeyer flasks containing 25 ml of the medium with the following compositions:

A	Standard	macroelement	solution	(Table 4)
B	"	"	"	x 2
C	"	"	"	with x 2 Mg
D	"	"	"	" x 2 K
E	"	"	"	" x 2 Ca
F	"	"	"	" x 2 NO_3
G	"	"	"	" x 2 PO_4
H	"	"	"	" x 2 SO_4

After incubation for 4 weeks in light at 26±2°C, the growth measurements were made and the results obtained are presented in Table 17 and Figure 12.

As found in the previous experiment, the growth enhanced when the level of macroelement salts was doubled. The increments in growth was measured by fresh and dry weights and total cell number on this medium (B) was

Table 17: Effect of the Total concentration of the Macroelement salts and that of Individual ions of these salts on Growth of *Datura* Callus cultures.

Inoculum : 100±10 mg of tissue (Dry weight: 6.0 mg and Total Cell No.0.04 $x10^6$) in 25 ml of Datura medium modified as mentioned in the legend of Figure 12.

Incubation : 4 weeks in light at 26±2°C.

Medium Composition	Fresh Wt (mg)	Dry Wt (mg)	Total Cell No. ($x\ 10^6$)
A	5266 (45)	228 (5.0)	2.2 (0.042)
B	6225 (55)	268 (3.8)	2.8 (0.053)
C	5386 (36)	236 (3.0)	2.2 (0.045)
D	5046 (37)	218 (5.5)	2.1 (0.022)
E	5402 (59)	225 (5.0)	2.3 (0.052)
F	6252 (52)	262 (4.0)	2.8 (0.048)
G	4462 (52)	198 (2.5)	1.9 (0.032)
H	4217 (36)	182 (2.0)	1.8 (0.036)

* Figures in the parentheses represent standard error.

Fig. 12. Effect of the Total concentration of the Macroelement salts and that of Individual ions of these salts on Growth of Datura Callus cultures.

100 mg tissue inoculated in:-

A	Standard	macroelement	solution	(Table 4)	
B	"	"	"	x 2	
C	"	"	"	with x 2	Mg
D	"	"	"	" x 2	K
E	"	"	"	" x 2	Ca
F	"	"	"	" x 2	NO_3
G	"	"	"	" x 2	PO_4
H	"	"	"	" x 2	SO_4

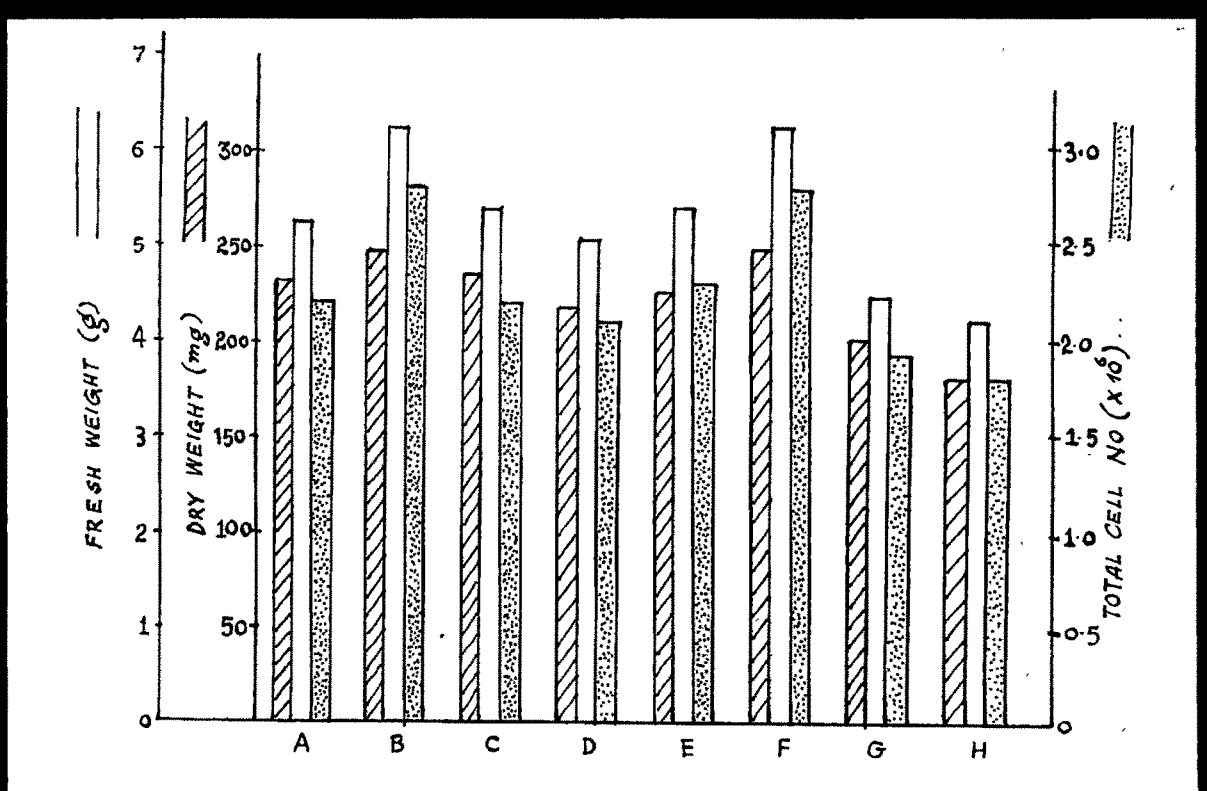

FRESH WEIGHT (g)
DRY WEIGHT (mg)
TOTAL CELL NO (x10⁶)
0
1
2
3
4
5
6
7
50
100
150
200
250
300
0·5
1·0
1·5
2·0
2·5
3·0
A
B
C
D
E
F
G
H

Fig. 12

62, 53 and 61 folds respectively as compared to 53, 45 and 55 folds obtained on standard (Table 4) medium. Data of growth attained on media in which the concentrations of separate macroelement ions were offered, clearly showed that doubling the concentration of the nitrate ion alone reproduced the effect of doubling the concentration of the whole of macroelement solution. Increasing the concentration of phosphate and sulphate ions resulted in marked reduction in growth. Doubling the concentration of potassium, magnesium and calcium ions did not however, produce any appreciable change in growth when compared with the growth attained on standard Datura medium. These results indicated that the level of nitrate in a fixed volume of the medium was limiting the growth, particularly cell division of the Datura tissue.

C. Effect of Higher Levels of Nitrate on Growth of Datura Callus tissue during Three Culture passages:

Whether the enhanced growth of Datura callus on increased nitrate medium was maintained during subsequent passages of cultures was examined. Measured amount of tissues (100±10 mg) grown on Datura medium (Table 4) were transferred separately to Erlenmeyer flasks

containing 25 ml of the following culture media:

A. Datura medium without nitrate

B. Datura medium (with standard nitrate supply)

C. Datura medium + 1 macroelement solution (double level of complete macroelement salts)

D. Datura medium + 1 fold extra nitrate ion (with double nitrate level)

E. Datura medium + 3 fold extra nitrate ions (with four-fold nitrate level)

After incubation for 4 weeks in light at 26±2°C growth was measured in each of the treatments and weighed amount of callus tissues 100±10 mg by fresh weight, from each of the treatments were transferred to the corresponding freshly prepared media of the same composition. This process was repeated twice and the results obtained were presented in Table 18.

It was clear from the data that the medium devoid of nitrate (A) could promote little growth during the first passage and drastic reduction was observed during subsequent culture passages. The growth of tissues on standard Datura medium (B), however, remained more or less constant during all the three culture passages (44 fold increase in dry weight and 55 fold increase in total

Table 18 : Effect of Higher Levels of Nitrate on Growth of *Datura* Callus tissues during Three Culture Passages

Inoculum : 100±10 mg of tissue (Dry weight: 6.0 mg and Total Cell No. 0.04×10^6 in 25 ml of

A. Datura medium minus nitrate
B. Datura medium (Table 4)
C. Datura medium x 2 Macroelement salts
D. Datura medium x 2 nitrate
E. Datura medium x 4 nitrate

Incubation : 4 weeks in light at 26±2°C.

Medium	First Passage		Second Passage		Third Passage	
	Dry Wt (mg)	Total Cell No. ($\times 10^6$)	Dry Wt (mg)	Total Cell No. ($\times 10^6$)	Dry Wt (mg)	Total Cell No. ($\times 10^6$)
A	112 (2.2)	1.0 (0.052)	76 (1.2)	0.8 (0.032)	42 (2.0)	0.5 (0.012)
B	221 (3.5)	2.2 (0.051)	229 (7.0)	2.3 (0.064)	218 (5.7)	2.2 (0.053)
C	225 (4.5)	2.7 (0.064)	224 (5.2)	2.3 (0.052)	147 (3.7)	1.2 (0.024)
D	249 (4.0)	2.7 (0.043)	226 (4.7)	2.3 (0.056)	142 (4.5)	1.1 (0.032)
E	228 (2.0)	2.4 (0.032)	72 (3.5)	0.8 (0.046)	70 (1.7)	0.6 (0.024)

* Figures in the parentheses represent standard error.

cell number). Growth attained on medium C (containing double doze of complete macroelement solution) and medium D (which had double nitrate level) showed almost similar high values in the first passage, thus confirming the results obtained in the earlier experiment. On the same media, however, pronounced decline in growth was observed in the subsequent culture passages, particularly in the third passage. Growth recorded on medium E (containing 4 fold nitrate) during the first passage compared favourably with that on standard Datura medium B. However, during the second passage there was marked reduction, the growth values registered being as low as those on the medium A which completely lacked the nitrate supply.

EXPERIMENT 9A: Effect of Nitrates on Growth of Datura Callus cultures

To determine the ability of callus tissues of Datura metel L. to utilize different forms of inorganic nitrogen, weighed amount (100±10 mg) of callus pieces were separately inoculated in 25 ml of Datura medium (Table 4) containing equimolar concentration of sodium nitrate (Medium A), potassium nitrate (Medium B), calcium nitrate (Medium C) and ammonium nitrate (Medium D) as nitrogen sources. Prior to incubation of this experiment

the callus tissues were grown on nitrogen free medium for a period of two weeks.

The growth responses of callus cultures, incubated for 4 weeks in light at 26±2°C to various nitrate supplements are presented in Table 19 and Figure 13. Tissues cultured on media containing sodium and potassium nitrates showed more or less similar growth as measured by all the three parameters (about 53 fold increase in fresh weight, 35 fold increase in dry weight and 55 fold increase in total cell number). Tissues grown on calcium nitrate medium showed marked increase in dry weight (38 fold), but a slight decline in total cell production (about 50 fold). There was pronounced enhancement in growth as measured by increase in fresh weight (62 fold) and total cell number (64 fold) when the nitrogen was supplied as ammonium nitrate at the same concentration (Medium D). However, dry weight of the tissues on this medium D showed much less increase (32 fold) as compared to corresponding values registered on the other three media.

Table 19 : Effect of Nitrates on Growth of *Datura* Callus cultures*

Inoculum : 100±10 mg tissue (Dry weight: 6.1 mg and Total Cell No. 0.04 x 10^6) in 25 ml of Datura medium with: Sodium nitrate (Medium A), Potassium nitrate (Medium B), Calcium nitrate (Mediun C) or Ammonium nitrate (Medium D) as nitrogen sources.

Incubation : 4 weeks in light at 26±2°C.

Medium	Fresh Wt (mg)	Dry Wt (mg)	Total Cell No. (x 10^6)
A	5440	217	2.25
	(25)	(2.8)	(0.042)
B	5263	210	2.15
	(74)	(1.8)	(0.048)
C	5023	232	2.00
	(87)	(2.5)	(0.052)
D	6192	196	2.55
	(37)	(2.5)	(0.061)

* Figures in the parentheses represent standard error.

Fig. 13. Effect of Nitrates on Growth of *Datura* Callus cultures.

100 mg tissue inoculated in Datura medium containing either $NaNO_3$(A), KNO_3(B), $Ca(NO_3)_2$(C) or NH_4NO_3(D) as nitrogen sources.

Other experimental details as given in Table 19.

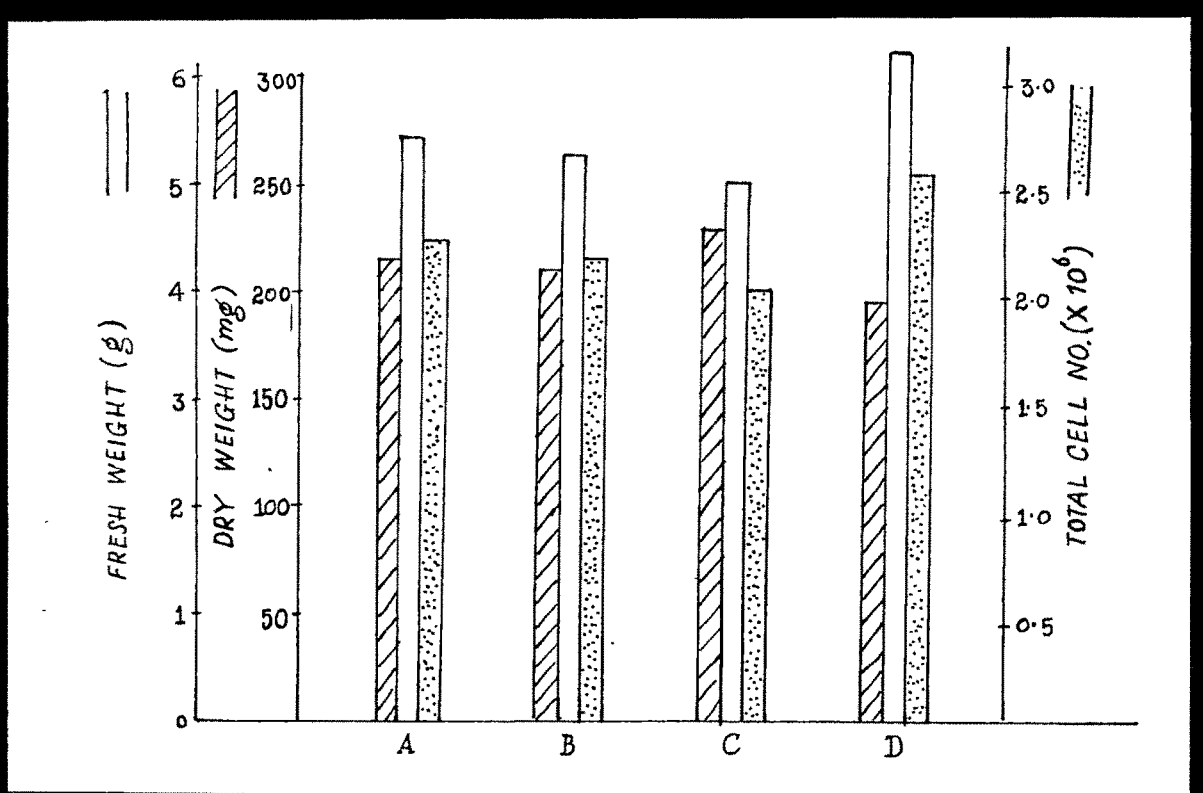

FRESH WEIGHT (g)
DRY WEIGHT (mg)
TOTAL CELL NO. (X 10⁶)
0
1
2
3
4
5
6
50
100
150
200
250
300
0·5
1·0
1·5
2·0
2·5
3·0
A
B
C
D

Fig. 13

B. Effect of addition of Casein hydrolysate on Growth of Datura Callus cultures:

The callus cultures of Datura were transferred to Datura medium (Table 4) devoid of casein hydrolysate for two weeks before inoculation of this experiment. Weighed amounts (100±10 mg) of callus tissues were transferred aseptically to Erlenmeyer flasks containing 25 ml of Datura medium (without casein hydrolysate) supplemented with 0.0, 150, 300, 600 or 1200 mg/l casein hydrolysate. Ten replicates were made for each treatment and the culture vessels were incubated for 4 weeks in light at 26±2°C. Final growth measurements are presented in Table 20 and Figure 14.

Absence of casein hydrolysate from the standard medium showed reduction in growth as measured by all the three parameters when compared to the tissues grown on Datura medium. Fresh weight and total cell number showed about 47 fold increase, while dry weight registered 26 fold increase. There was general enhancement in growth with increasing levels of casein hydrolysate in the nutrient medium.

As there was no appreciable increase in dry weight of the tissues when very high concentration of

Table 20 : Effect of Casein hydrolysate on Growth of *Datura* Callus cultures*

Inoculum : 100±10 mg of tissue (Dry weight: 6.0 mg and Total Cell No. 0.04 x 10^6) in 25 ml of *Datura* medium (without casein hydrolysate) supplemented with 150, 300, 600 or 1200 mg/l casein hydrolysate.

Incubation : 4 weeks in light at 26±2°C.

Casein hydrolysate (mg/l)	Fresh Wt (mg)	Dry Wt (mg)	Total Cell No. (x 10^6)
0.0	4791 (41)	159 (4.5)	1.85 (0.025)
150	5056 (75)	191 (4.0)	2.10 (0.012)
300	5552 (50)	216 (5.0)	2.30 (0.025)
600	6362 (87)	224 (5.7)	2.45 (0.042)
1200	6663 (63)	226 (3.2)	2.55 (0.044)

* Figures in the parentheses represent standard error.

Fig. 14. Effect of casein hydrolysate on Growth of *Datura* Callus cultures.

100 mg of tissue in 25 ml of Datura medium supplemented with various concentrations of casein hydrolysate.

Other experimental details as given in Table 20.

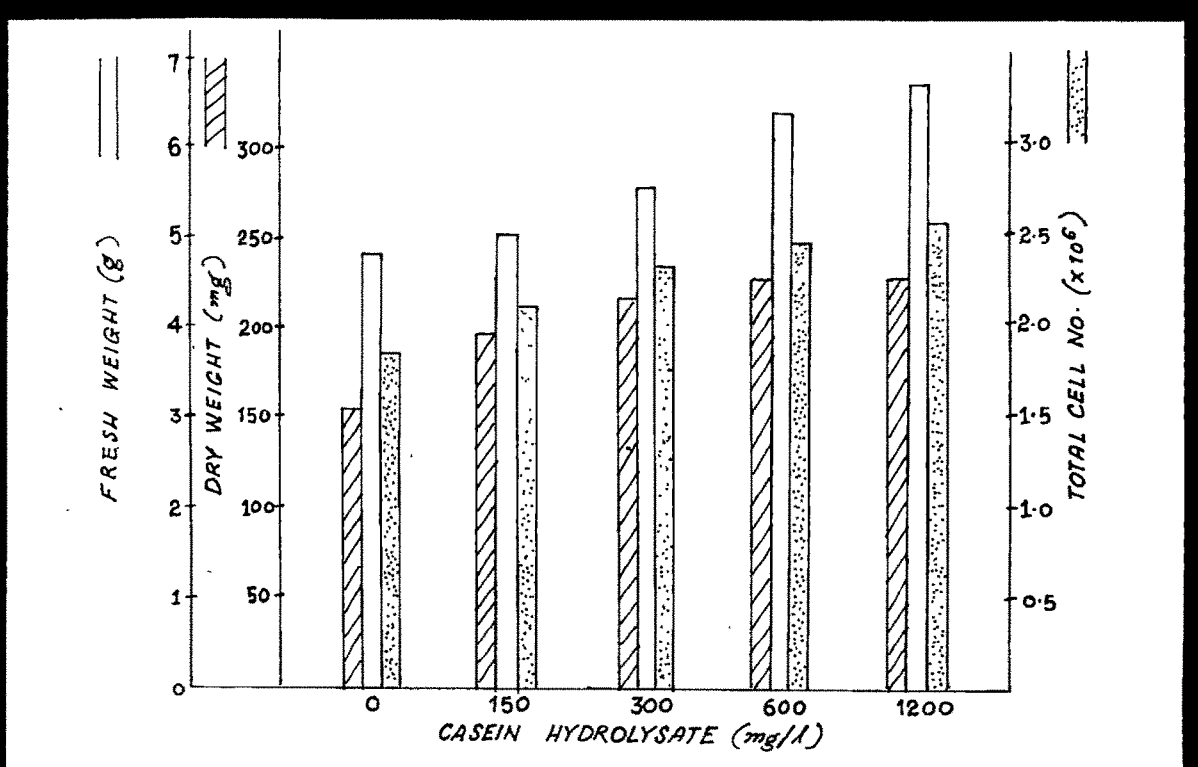

FRESH WEIGHT (g)
DRY WEIGHT (mg)
TOTAL CELL NO. (x10⁶)
0
1
2
3
4
5
6
7
50
100
150
200
250
300
0·5
1·0
1·5
2·0
2·5
3·0
0
150
300
600
1200
CASEIN HYDROLYSATE (mg/l)

Fig. 14

casein hydrolysate was incorporated in the medium, for routine growth of Datura tissues 300 mg/l casein hydrolysate was added to the nitrate containing medium as given in Table 4.

EXPERIMENT 10 : Effect of Molybdenum on Growth of Datura Callus cultures

To determine the effect of various levels of molybdenum on the growth of Datura callus, uniform (100±10 mg) inocula of tissues were separately transferred in Erlenmeyer flasks containing 25 ml of Datura medium (Table 4) supplemented with 0.0, 0.001, 0.01, 0.1 or 1.0 mg/l molybdenum. Ten replicates were made for each treatment. After incubation for 4 weeks in light at 26±2°C, growth measurements were made as described in Materials and Methods, 5A, B and C.

The results presented in Table 21 and Figure 15 showed that maximum total cell number (57 fold) and highest increase in fresh weight (59 fold) and dry weight (39 fold) were registered in the medium containing 0.001 mg/l molybdenum. Further increase in molybdenum concentration resulted in corresponding decrease in growth measured by all the three parameters. Higher levels of molybdenum (1.0 mg/l) was found to be toxic for growth of Datura tissue.

Table 21 : Effect of Molybdenum on Growth of Datura Callus cultures*

Inoculum : 100±10 mg of tissue (Dry weight: 6.1 mg and Total Cell No. 0.04 x 10^6) in 25 ml of Datura medium supplemented with 0.001, 0.01, 0.1 or 1.0 mg/l molybdenum.

Incubation : 4 weeks in light at 26±2°C.

Molybdinum (mg/l)	Fresh Wt (mg)	Dry Wt (mg)	Total Cell No. (x 10^6)
0.0	5092	207	2.0
	(62)	(1.2)	(0.021)
0.001	5883	237	2.28
	(37)	(3.4)	(0.043)
0.01	5268	208	2.05
	(39)	(2.2)	(0.042)
0.1	5049	202	2.00
	(47)	(4.2)	(0.051)
1.0	4540	184	1.82
	(64)	(4.0)	(0.042)

* Figures in the parentheses represent standard error.

Fig. 15. Effect of Molybdenum on Growth of *Datura* Callus cultures.

100 mg of tissue in 25 ml of Datura medium supplemented with various concentrations of molybdenum.

Other experimental details as given in Table 21.

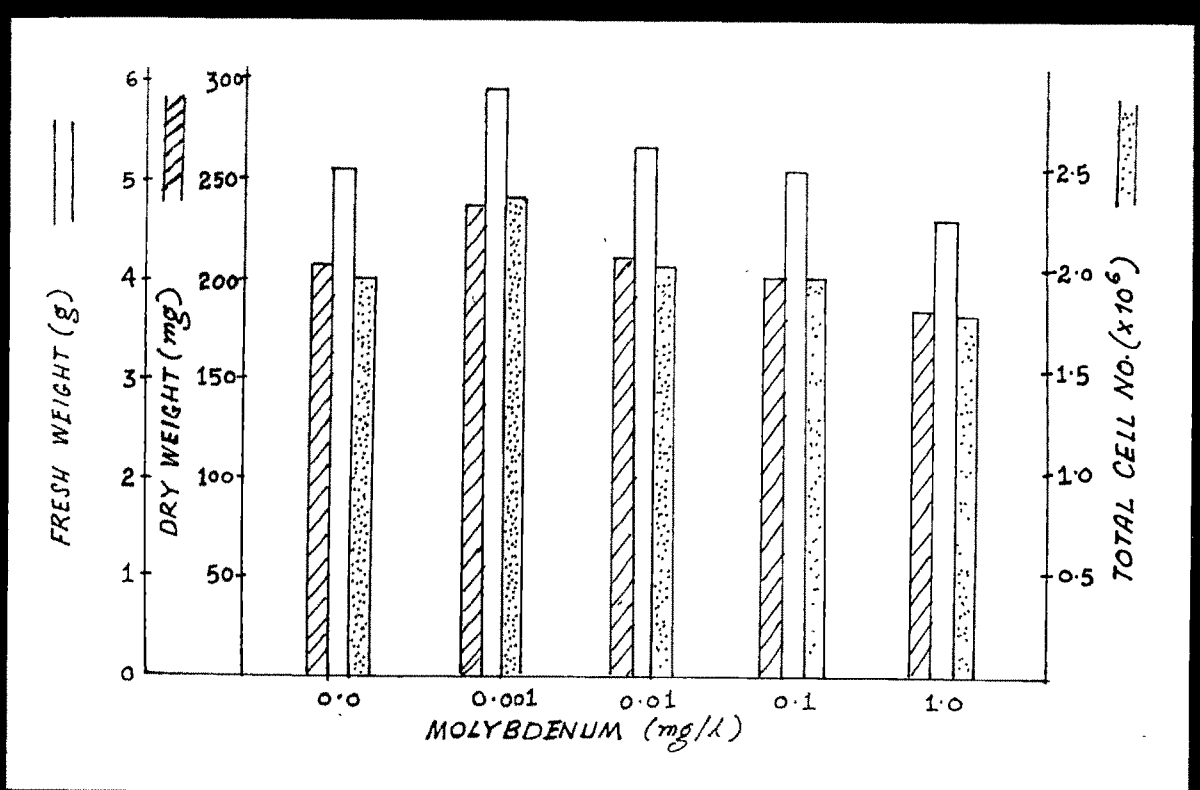

FRESH WEIGHT (g)
DRY WEIGHT (mg)
TOTAL CELL NO. (x10⁶)
0
1
2
3
4
5
6
50
100
150
200
250
300
0.5
1.0
1.5
2.0
2.5
0·0
0·001
0·01
0·1
1·0
MOLYBDENUM (mg/l)

Fig. 15

DISCUSSION

Callus was induced on excised segments of mature anthers of Datura metel in a 2,4-D containing medium (Experiment 1). Initiation of callus was more rapid on the medium which also contained coconut milk and casein hydrolysate. Histological examination revealed that the callus originated mainly from the connective region of the anther.

Of the auxins tested, 2,4-D at 2.0 mg/l concentration supported the highest growth of anther callus tissues (Experiment 2). Studies on the effect of kinetin, alone and in combination with 2,4-D, on growth of Datura tissues indicated that the growth increased with increasing level of kinetin from 0 to 0.5 mg/l (Experiments 6A and B). At the optimal combination of kinetin (0.5 mg/l) and 2,4-D (2.0 mg/l) the effect on growth was found to be addative.

Of the sugars tested, sucrose was found to be the most efficient energy source for growth of Datura callus. Glucose, fructose, equimolar mixture of glucose and fructose and maltose supported almost equal amount of growth; which, however, was poor compared to growth supported by sucrose. The growth attained on the medium

containing soluble starch was the least; being about half as much as that obtained with sucrose (Experiment 5A). Further, growth measured by increase in dry weight and total cell number enhanced with increasing levels of sucrose from 0 to 2 per cent. Though there was reduction in growth at higher levels of sucrose, the dry weight per cell increased with increasing concentration of sucrose (Experiment 5B).

The results obtained in Experiment 7 showed that the growth of Datura callus was markedly influenced by inoculum/volume ratio. In a fixed volume of the medium, the highest growth was attained with the smallest (50 mg) of the inoculum sizes tested (Experiment 7A). Conversely, the growth of a fixed inoculum size increased with increasing volumes of the medium (Experiment 7B). This close relationship between the inoculum/volume ratio and the final growth attained strongly suggested that growth was limited by the supply of some essential nutrient. This limiting nutrient was neither sucrose (Experiment 5B) nor one of the microelement salts (Experiment 8A); because when their levels were raised in the medium growth was not enhanced. However, doubling the concentration in the medium of the macroelement salts caused marked improvement in growth. When the

concentrations of the individual ions of these salts were altered, it was found that the supply of nitrate was limiting the growth of Datura callus tissue.

The growth of Datura tissues incubated in the medium which lacked nitrate was, therefore, very poor and the growth improved when the level of nitrate supply was raised (Experiment 8C). This enhanced growth, however, was not maintained during subsequent passages. The growth attained was about equal when the nitrate was supplied either in the form of sodium, calcium or potassium salts. However, when ammonium nitrate was supplied as nitrogen source there was appreciable growth promotion. Addition of casein hydrolysate to the nitrate containing medium further enhanced growth (Experiment 9). Incorporation of molybdenum at 0.001 mg/l brought about marked increase in growth; while higher levels of molybdenum proved toxic (Experiment 10).