CHAPTER IV

PHYSIOLOGICAL AND BIOCHEMICAL STUDIES IN DATURA CALLUS CULTURES

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The results obtained in Experiment 5 (Chapter III) clearly showed that sucrose was more effective source of carbon and energy for the growth of Datura callus cultures than any other source tested. To elucidate the superiority of sucrose, experiments were designed to study the effect of different carbohydrates on total and free sugars as well as on the activity of invertase in Datura callus tissues. Similarly, experiments were conducted to examine the differences (if any) in the patterns of cellular nitrogen and glutamic oxaloacetic transaminase activity in Datura tissues grown on sucrose or glucose media.

Moreover, it was observed in the previous Chapter (Experiments 7 and 8) that the growth of Datura callus was limited by nitrate supply in the medium. Growth enhanced when the level of nitrate was increased and also when casein hydrolysate as well as molybdenum were added to the nitrate containing medium. To pursue this line of investigation further, experiments were planned to examine the changes in the patterns of

nitrate reductase and glutamic oxaloacetic transaminase activities in Datura tissue cultures.

Finally, it was felt relevant to study the acid phosphatase activity also in Datura callus tissues. Experiments performed with above objectives are described in the present Chapter.

EXPERIMENT 11 : Effect of different Carbohydrates on Total, Free sugars and on Invertase activity of Datura Callus cultures

Earlier studies on carbohydrate nutrition (Experiment 5, Chapter III) revealed that out of the carbohydrates tested, sucrose was found to be the best energy source. In the present experiment detailed studies were carried out to examine the activity of enzyme invertase and the levels of total and free sugars present in the tissues grown on media containing different carbohydrates.

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

A	Datura medium	minus	sugar	
B	"	"	with	2% sucrose (Table 4)
C	"	"	"	2% glucose
D	"	"	"	2% fructose
E	"	"	"	2% equimolar mixture of glucose-fructose
F	"	"	"	2% Maltose
G	"	"	"	2% soluble starch

Prior to the inoculation of this experiment the callus tissues were grown on sugar free medium for a

period of two weeks to minimise carry-over effect. The cultures were incubated in light at 26±2°C for 21 days. A fixed number of replicates was harvested every third day after inoculation and about 1.5 grams of tissues were collected from each of the treatments for the enzyme assay. The procedure followed for estimation of invertase activity is described in Materials and Methods, 8B. About one gram of the tissue was collected from each treatment, and after drying in an oven at 60°C for 48 hours, the tissues were used for the estimation of total and free sugars by Nelson's (1955) method as described in Materials and Methods, 6A.

The results presented in Tables 22 and 23 showed that the total and free sugars increased throughout the growth period in all the treatments including the control. The increase in sugars per gram dry weight were maximum on 15th day in all the treatments and declined thereafter. The ratio between the total and free sugars also increased from 0 to 15th day and declined thereafter. The bound sugars (total sugars-free sugars) also followed the same pattern as that of total sugars. Of all the carbohydrates tested,

the tissues grown on sucrose medium contained high per cent of total and bound sugars and less of free sugars when compared with the tissues grown on other carbohydrates. The tissues grown on glucose, fructose, glucose fructose 1:1 mixture and maltose contained almost equal amounts of the total and free sugars. Soluble starch showed the lowest amount of total and free sugars during the course of culture.

The activity of enzyme invertase increased with the age of the tissue cultures grown on sucrose, glucose or fructose containing media upto day 15 after which it declined. The invertase activity was appreciably higher in the tissues grown on media containing sucrose than in those grown on glucose or fructose. Though the peak of activity was attained on day 15, the rate of increase in enzyme activity was highest in all the three treatments between day 9 and day 12. The enzyme activity was recorded twice as much as in tissues grown on sucrose containing medium on day 15 than the corresponding values obtained in tissues incubated on glucose or fructose media.

The enzyme activity and growth by increase in dry weight of Datura anther callus grown on sucrose containing medium, is illustrated in Figure 16. The

invertase activity rose rapidly during the first two weeks of culture during which period increase in dry weight was not very high. Pronounced increase in dry weight was recorded during the third week when the invertase activity was declining.

Growth measured by increase in dry weight was maximum in tissues incubated on sucrose containing medium and was most poor in tissues grown in media which lacked sugar. Glucose, fructose, glucose-fructose mixture or maltose in media supported almost equal amount of growth of the callus tissues; while growth of tissues grown on soluble starch was relatively much less. Initially, the tissues contained more of free (reducing) sugars than bound (non-reducing) sugars and the same pattern was observed in the control as well as in tissues grown on all other carbohydrates except sucrose medium—where the amount of non-reducing sugars was higher than the reducing sugars all through the course of culture for 21 days. This might probably be responsible for more growth on sucrose containing medium and perhaps explains more efficiency of sucrose as energy source over other carbohydrates tested.

Table 24 : Effect of different Carbohydrates on Invertase activity of Datura Callus cultures

Inoculum : 100±10 mg of tissue in 25 ml of Datura medium (without sucrose) supplemented with 2% sucrose, 2% glucose or 2% fructose.

Incubation : 21 days in light at 26±2°C.

Time (days)	Specific activity* of Enzyme carbohydrate source		
	Sucrose	Glucose	Fructose
0	3.6	3.6	3.6
3	6.0	4.2	4.0
6	8.6	5.2	5.0
9	12.8	6.4	6.2
12	19.6	10.5	10.0
15	25.4	12.5	12.2
18	18.4	8.6	7.9
21	14.5	6.3	6.1

Enzyme Units : Amount of enzyme liberating 1 microgram of reducing sugar in 60 minutes at 37°C = 1 unit.

*Specific Activity : Number of enzyme units per mg protein.

Fig. 16. Progress of Growth and Invertase activity in Datura Callus during the course of culture.

Experimental details as given in Tables 22 and 24.

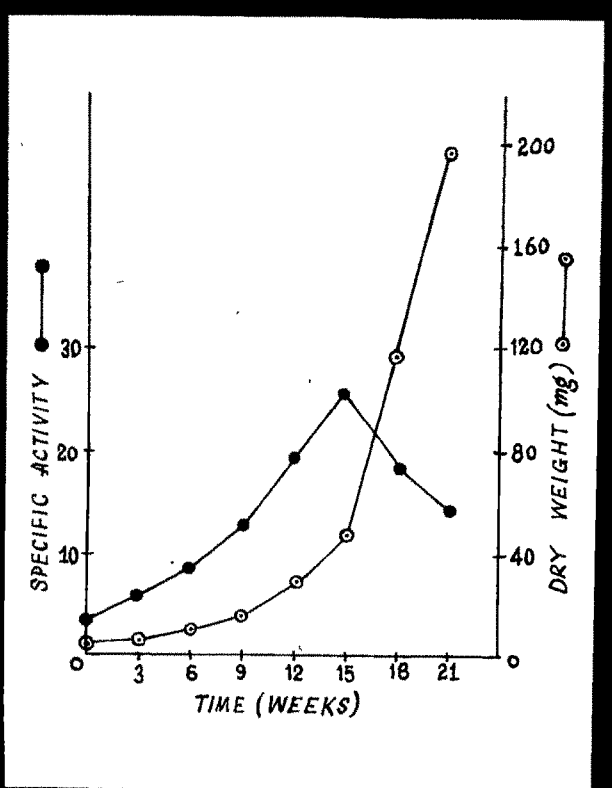
SPECIFIC ACTIVITY
30
20
10
0
3
6
9
12
15
18
21
TIME (WEEKS)
200
160
120
80
40
0
DRY WEIGHT (mg)

Fig. 16

EXPERIMENT 12 : Effect of Sucrose on Total, Free sugars and Invertase activity of Datura Callus cultures

Experiment was next conducted to find out the changes in the total and free sugar content as well as changes in the invertase activity with changing sucrose concentration in the culture medium.

Tissue pieces of Datura weighing 100±10 mg each were transferred separately to Erlenmeyer flasks containing 25 ml of Datura medium without sucrose or supplemented with 1.0, 2.0 or 4.0 per cent sucrose. Pre-incubation of callus tissues was done for two weeks on sucrose free medium to minimise carry-over. The cultures were incubated in light at 26±2°C for 21 days. Every third day after inoculation a fixed number of replicates was harvested and 1.5 grams of tissue collected from each treatment for the enzyme assay (Materials and Methods, 8B). About one gram of tissue collected from each treatment and dried in an oven for 48 hours at 60°C was used for the analysis of total and free sugars (Materials and Methods, 6A).

Data presented in Tables 25 and 26 indicated that the dry weight increased with the increase in sucrose

Table 25 : Effect of Sucrose on Growth and on Total and Free sugar contents in Datura Callus cultures*

Inoculum : 100±10 mg of tissue in 25 ml of Datura medium with sucrose or supplimented with 1.0, 2.0 or 4.0 per cent sucrose.

Incubation : 21 days in light at 26±2°C.

Time (days)	Sucrose concentration (%)											
	0.0			1.0			2.0			4.0		
	Dry Wt (mg)	Total sugar (mg)	Free sugars (mg)	Dry Wt (mg)	Total sugars (mg)	Free sugars (mg)	Dry Wt (mg)	Total sugars (mg)	Free sugars (mg)	Dry Wt (mg)	Total sugars (mg)	Free sugars (mg)
0	5.0	2.10	1.10	5.0	2.10	1.10	5.0	2.10	1.10	5.0	2.10	1.10
3	5.2	2.20	1.15	6.0	2 64	1.28	6.4	2.89	1.42	6.5	3.02	1.48
6	5.9	2.47	1.35	8.4	3.78	1.85	9.2	4.45	2.12	9.0	4.50	2.19
9	8.2	3.67	1.81	14.6	7.38	3.34	15.6	9.36	3.76	15.5	10.73	4.03
12	9.6	4.39	2.32	22.0	12.49	5.3	28.0	19.48	7.89	26.5	20.72	7.52
15	12.2	5.73	3.02	40.0	24.80	10.24	47.0	35.20	14.85	46.0	37.61	13.84
18	18.0	8.15	4.32	102.6	57.46	26.67	118.4	77.91	35.89	116.0	79.23	32.59
21	24.5	10.58	6.76	148.0	71.00	31.08	196.0	108.40	47.62	184.0	109.29	40.48

* Data represent average of eight replicates.

Table 26 : Effect of Sucrose on Total and Free sugars present in unit dry weight of *Datura* Callus cultures*

Inoculum : 100±10 mg of tissue in 25 ml of Datura medium either without sucrose or supplemented with 1.0, 2.0, or 4.0 per cent sucrose.

Incubation : 21 days in light at 26±2°C.

Time (days)	Sucrose concentration (%)											
	0			1			2			4		
	Total sugars	Bound sugars	Free sugars	Total sugars	Bound sugars	Free sugars	Total sugars	Bound sugars	Free sugars	Total sugars	Bound sugars	Free sugars
0	420	200	220	420	200	220	420	200	220	420	200	220
3	429	207	222	440	226	214	453	230	223	465	237	228
6	444	215	229	450	229	221	484	254	230	498	254	244
9	448	216	232	506	277	229	600	359	241	628	368	266
12	462	220	242	568	327	241	696	413	382	712	428	284
15	470	222	248	620	364	256	750	436	316	756	455	301
18	453	213	240	560	300	260	658	358	303	683	402	281
21	432	197	235	480	270	210	533	310	243	594	374	220

* Data (mg) represent average of eight replicates.

Table 27 : Effect of Sucrose in the Medium on Invertase activity in Datura Callus cultures

Inoculum : 100±10 mg of tissue in 25 ml of Datura medium either without sucrose or supplemented with 1.0, 2.0 or 4.0 per cent sucrose.

Incubation : 21 days in light at 26±2°C.

Time (days)	Sucrose (per cent)	Specific activity* of Enzyme
0	0.0	3.6
3	0.0	3.8
	1.0	4.8
	2.0	6.0
	4.0	6.2
6	0.0	4.0
	1.0	5.6
	2.0	8.6
	4.0	8.8
9	0.0	4.6
	1.0	7.4
	2.0	12.8
	4.0	14.0
12	0.0	5.6
	1.0	14.2
	2.0	19.6
	4.0	21.5
15	0.0	5.2
	1.0	20.6
	2.0	25.4
	4.0	28.2
18	0.0	5.2
	1.0	15.8
	2.0	18.4
	4.0	20.6
21	0.0	4.4
	1.0	13.0
	2.0	14.5
	4.0	16.4

Enzyme Units : Amount of enzyme liberating 1 microgram of reducing sugar in 60 minutes at 37°C = 1 unit.

*Specific Activity : Number of enzyme units per mg protein.

concentration upto 2%, while at 4% sucrose the dry weight declined. But the total sugars and bound sugars increased with the increasing sucrose level. The ratio of bound sugars to the free sugars also increased with increasing concentration of sucrose in the medium.

The invertase activity presented in Table 27, increased with the age of the culture upto 15th day and declined thereafter in all the treatments. With the increasing level of sucrose from 0 to 4 per cent, the enzyme activity also enhanced.

EXPERIMENT 13 : Changes in Nitrogen content associated with Growth of *Datura* Callus cultures

Weighed pieces (100±10 mg) of callus tissues of *Datura* were inoculated in Erlenmeyer flasks containing 25 ml of Datura medium (Table 4) minus casein hydrolysate. The culture flasks were incubated in light at 26±2°C. Fixed number of replicates were harvested every third day after inoculation upto 21st day for the measurement of growth and for the estimation of their soluble and insoluble nitrogen contents. Nitrogen estimations were made as described in Materials and Methods, 6B.

It was clear from the Table 28 and Figure 17 that during the initial 12 days of culture there was a rapid rise in cell nitrogen content, especially insoluble nitrogen. The insoluble nitrogen content continued to increase at a higher rate upto 12th day followed by a sharp decline. The other growth parameters continued to increase after insoluble nitrogen became stationary. Soluble nitrogen increased sharply during first six days and after attaining the peak value on 12th day it declined gradually. It was also observed that the content of insoluble nitrogen rose steeply during the initial 12 days during which period dry weight and total cell number increased only slightly. Thus a pronounced rise in nitrogen per cell occured at a time well before the cells begin to devide rapidly and as the tissue embarked upon rapid cell division, the nitrogen content per unit dry weight as well as per cell declined.

Table 28: Changes in the Nitrogen content associated with Growth of *Datura* Callus cultures

Inoculum : 100±10 mg of tissues in 25 ml of Datura medium (minus casein hydrolysate).

Incubation : 21 days in light at 26±2°C.

Time (days)	Dry Wt (mg)	Total Cell No. ($\times 10^6$)	Nitrogen mg/g dry wt		Total Nitrogen per cell ($mg \times 10^{-6}$)
			Soluble	Insoluble	
0	5.0	0.04	1.8	6.2	0.10
3	6.4	0.05	6.2	10.3	0.21
6	9.2	0.08	10.8	18.2	0.33
9	15.6	0.12	11.6	30.0	0.54
12	28.0	0.15	13.4	40.6	1.00
15	47.0	0.38	12.8	26.8	0.48
18	118.4	0.95	10.5	25.0	0.44
21	196·0	1.57	10.0	22.0	0.40

Fig. 17. Changes in Nitrogen content associated with Growth of *Datura* Callus cultures.

100 mg of tissues in 25 ml of Datura medium (minus casein hydrolysate).

Other experimental details as given in Table 28.

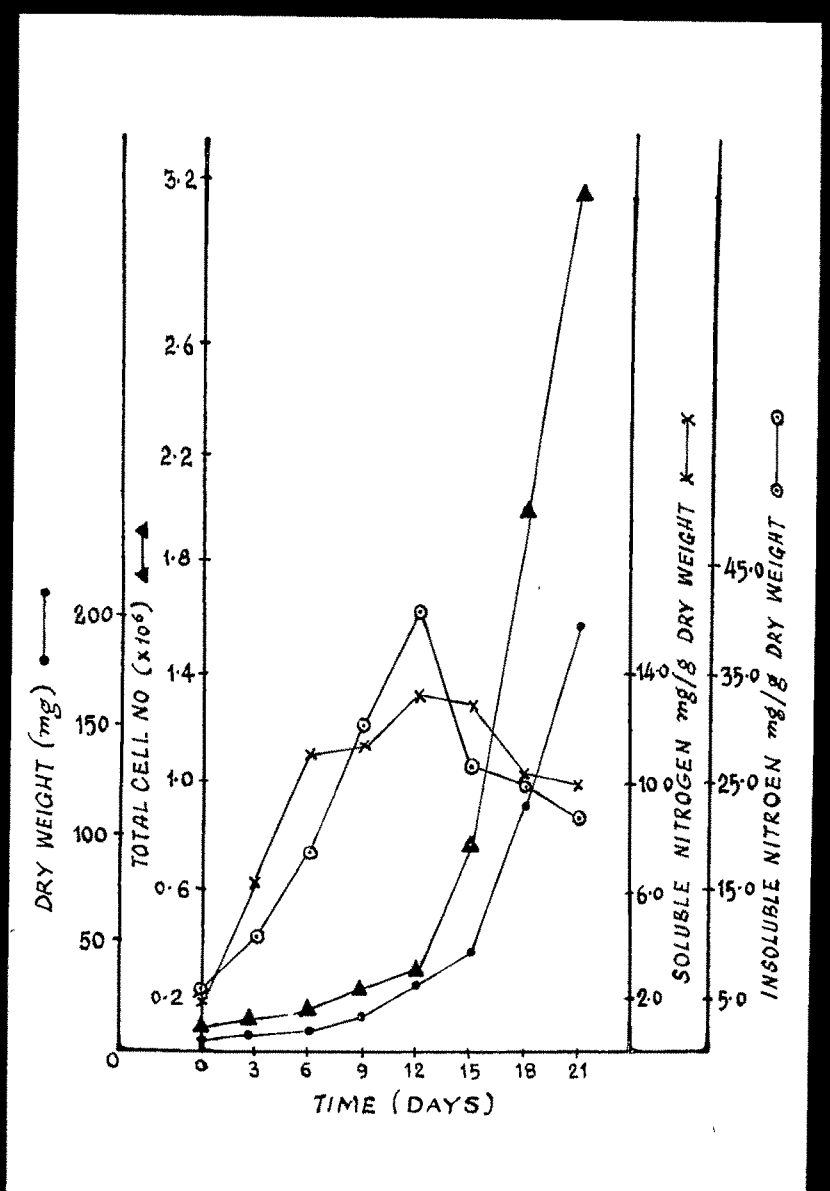
DRY WEIGHT (mg)
TOTAL CELL NO (x10⁶)
SOLUBLE NITROGEN mg/g DRY WEIGHT
INSOLUBLE NITROEN mg/g DRY WEIGHT
TIME (DAYS)
0
50
100
150
200
0·2
0·6
1·0
1·4
1·8
2·2
2·6
3·2
2·0
6·0
10·0
14·0
5·0
15·0
25·0
35·0
45·0
0
3
6
9
12
15
18
21

Fig. 17

EXPERIMENT 14 : Changes in the Nitrogen content of the Callus tissues of Datura grown on Sucrose or Glucose media

Experiment was next conducted to find out the relative amounts of cellular nitrogen present in Datura callus when grown in either sucrose or glucose containing media.

100±10 mg of tissue pieces of Datura were inoculated in Erlenmeyer flasks containing 25 ml of Datura medium (Table 4) minus casein hydrolysate. The culture flasks were incubated in light at 26±2°C for 21 days. A fixed number of replicates was harvested every third day after inoculation for the estimation of nitrogen contents (Materials and Methods, 6B).

From the data presented in Table 29 it was clear that the nitrogen content mg/g dry weight of the tissues increased with the age of the culture upto 12th day and declined thereafter in the tissues grown both on sucrose and glucose containing media. The callus tissues grown on sucrose medium contained more of nitrogen than those grown on glucose containing medium. It was evident that the total protein content of the

Table 29: Changes in Nitrogen content in the Callus cultures of Datura grown on Sucrose or Glucose containing media

Inoculum : 100±10 mg of tissue in 25 ml of Datura medium (minus casein hydrolysate) containing either 2% sucrose or 2% glucose.

Incubation : 21 days in light at 26±2°C.

Time (days)	Total nitrogen mg/g dry Wt	
	Sucrose medium	Glucose medium
0	8.0	8.0
3	16.5	15.0
6	29.0	22.2
9	41.6	36.5
12	54.0	44.0
15	39.6	37.1
18	35.5	32.5
21	32.2	29.0

callus tissues grown on sucrose medium was higher than that of glucose grown tissues.

EXPERIMENT 15: Effect of Sucrose and Glucose on Glutamic Oxaloacetic Transaminase in Datura Callus tissue during the course of culture

Earlier studies had shown that the growth attained by Datura anther callus on sucrose containing medium was higher than that registered on the glucose containing medium (Experiment 5, Chapter III). Further analysis of the tissues revealed that levels of proteins (Experiment 13, Chapter IV) and carbohydrates (Experiment 11, Chapter IV) were higher in tissues incubated on sucrose containing medium as compared with the corresponding levels in tissues incubated on glucose containing medium. This seemed to suggest that in sucrose grown tissues there was enhanced cellular synthesis. In view of the well recognised role of free amino acids in protein synthesis and the function of transamination systems in building up of the cellular pool of free amino acids the glutamic oxaloacetic transaminase (GOT) activity was measured in glucose grown and sucrose

Table 30: Effect of Sucrose and Glucose on Glutamic Oxaloacetic Transaminase in *Datura* Callus tissues during the course of Culture

Inoculum : 100±10 mg of tissues in 25 ml of Datura medium (minus casein hydrolysate) containing either 2% sucrose or 2% glucose.

Incubation : 21 days in light at 26±2°C.

Time (days)	Specific activity* of Enzyme	
	Sucrose medium	Glucose medium
0	not detectable	not detectable
3	9.5	5.8
6	14.8	9.2
9	20.9	12.6
12	28.6	16.8
15	25.4	14.0
18	15.2	9.2
21	10.0	6.5

Enzyme Units : Amount of enzyme produces 1 µ g of glutamate in 1 hour at 37°C.

*Specific Activity: Number of enzyme units per mg protein.

grown Datura callus tissues. The enzyme assay was done as described in Materials and Methods, 8C.

The data presented in Table 30 indicated that the activity of GOT increased with the age of the culture both in sucrose and glucose grown callus tissues from 0 to 12th day and started to decline thereafter. The GOT activity in sucrose grown tissues was significantly higher than that in glucose grown tissues throughout the growth period. This would probably account for higher protein content in sucrose grown Datura callus tissue.

EXPERIMENT 16 : Changes in the Nitrate Reductase (NR) and Glutamic Oxaloacetic Transaminase (GOT) activity in Datura Callus tissue during the course of culture

Experiments were conducted to find out the activity of enzyme nitrate reductase (NR) and glutamic oxaloacetic transaminase (GOT) during the course of culture. 100±10 mg of callus tissues growing on Datura medium without casein hydrolysate were transferred to Erlenmeyer flasks containing 25 ml of freshly made medium

of the same composition (i.e as given in Table 4 minus casein hydrolysate). The flasks were incubated in light at a constant temperature of 26±2°C. A fixed number of replicates was harvested every third day after inoculation upto 21st day. About 2 grams of tissue was collected from each of the treatments and used for nitrate reductase and glutamic oxaloacetic transaminase assays (Materials and Methods, 8C,D).

The results presented in Table 31 showed that though initially the activity of nitrate reductase was not detectable, it rose sharply during the first three days of culture. The peak value was attained on day 12 before it declined gradually. The specific activity of NR plotted against time in Figure 18 further confirmed that the enzyme was induced during the first three days; and that the rate of increase in activity slowed down considerably after day 6, though the peak value was recorded on day 12.

The activity of enzyme GOT simulated the same trend as that of NR during the course of culture period. The specific activity of the enzyme plotted as function of culture age in Figure 18 indicated that the

Table 31: Changes in the Nitrate Reductase and Glutamic Oxaloacetic Transaminase activities in *Datura* Callus tissues during the course of Culture

Inoculum : 100±10 mg of tissue in 25 ml of Datura medium (minus casein hydrolysate).

Incubation : 21 days in light at 26±2°C.

Time (days)	Specific activity* of Enzyme	
	Nitrate reductase	Glutamic oxaloacetic transaminase
0	Not detectable	Not detectable
3	8.2	9.5
6	14.5	14.8
9	17.8	20.9
12	22.6	28.6
15	16.4	25.4
18	12.5	15.2
21	8.0	10.0

Enzyme Units

GOT : Amount of enzyme produces 1 µ g of glutamate in 1 hour at 37°C.

NR : Amount of enzyme which can reduce 1 mµ mole of substrate in 1 minute.

*Specific Activity : Number of enzyme unit per mg protein.

Fig. 18. Changes in Nitrate Reductase (NR) and Glutamic Oxaloacetic Transaminase (GOT) activities in Datura Callus tissues during the course of culture.

100 mg of tissue in 25 ml of Datura medium (minus casein hydrolysate).

Other experimental details as given in Table 31.

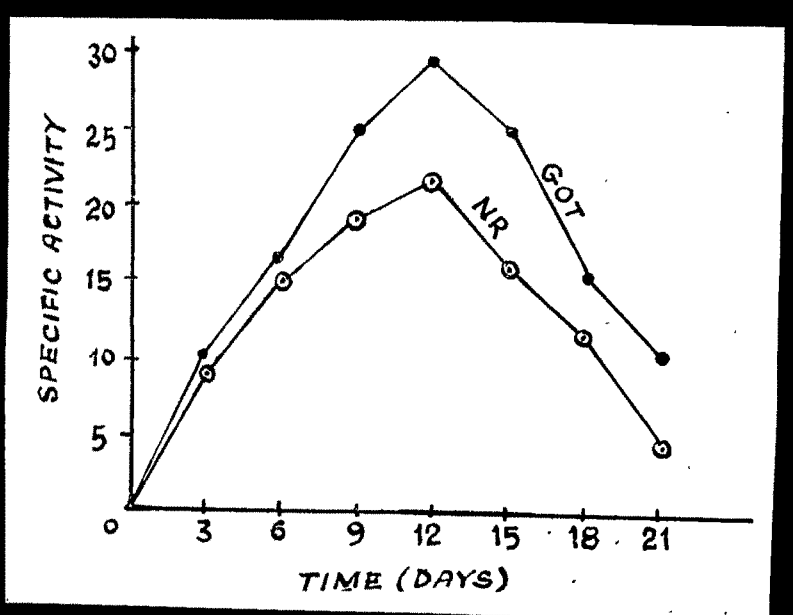
SPECIFIC ACTIVITY
30
25
20
15
10
5
0
3
6
9
12
15
18
21
TIME (DAYS)
GOT
NR

Fig. 18

activity of enzyme was entirely induced during the first three days. It increased with the age of the culture upto 12th day and declined thereafter.

EXPERIMENT 17 : Effect of Nitrate Level in the Medium on Nitrate Reductase and Glutamic Oxaloacetic Transaminase activities in Datura Callus tissues

Measured amounts of tissues (100±10 mg) were transferred to Erlenmeyer flasks containing 0, 1, 2 or 4 folds nitrate level present in Datura medium (minus casein hydrolysate). Prior to ~~incubation~~ inoculation of the tissue ~~for~~ experiment the were incubated for a period of two weeks ~~was done~~ in Datura medium which lacked casein hydrolysate and nitrate to minimise the carry-over effect. The callus cultures were incubated in light at a constant temperature of 26±2°C for 21 days. A fixed number of replicates was harvested every third day after inoculation. The tissues from each treatment were pooled separately for the enzyme assays as described in Materials and Methods, 8C,D.

The results presented in Table 32 showed that the nitrate reductase activity was not detectable in

tissues grown on media lacking nitrate and that the NR activity increased with the increase in the level of nitrate ion in the medium. Earlier it was found that higher levels (4 fold) of nitrate inhibited the growth (Experiment 8, Chapter III). It was observed in the present experiment, however, even at this higher level of nitrate in the medium, there was enhancement in activity of the enzyme nitrate reductase.

Data concerning GOT activity presented in Table 33 revealed that the activity increased with the increase in nitrate concentration upto double the level of nitrate. Further addition of nitrate caused, however, pronounced reduction in the enzyme activity. The pattern of GOT activity thus showed a trend similar to the growth pattern of Datura tissue at various nitrate levels as observed in Experiment 8, Chapter III.

Table 32: Effect of Nitrate level in the Medium on the Nitrate Reductase activity in *Datura* Callus cultures

Inoculum : 100±10 mg of tissue in 25 ml of Datura medium (minus casein hydrolysate) containing either no nitrate or with 1, 2 or 4 fold nitrate level.

Incubation : 21 days in light at 26±2°C.

Time (days)	Specific activity* of Enzymes Nitrate concentration (x folds)			
	0	1	2	4
0	Not detectable	Not detectable	Not detectable	Not detectable
3	"	8.2	12.6	16.8
6	"	14.5	18.2	24.3
9	"	17.8	23.8	30.2
12	"	22.6	28.4	36.5
15	"	16.4	21.0	28.3
18	"	12.5	18.2	20.2
21	"	8.0	12.4	16.4

Enzyme Units : Amount of enzyme which can reduce 1 m μ mole of substrate in one minute.

*Specific Activity: Number of enzyme units per mg protein.

Table 33: Effect of Nitrate level in Medium on the Glutamic Oxaloacetic Transaminase activity in *Datura* Callus cultures

Inoculum : 100±10 mg of tissue in 25 ml of Datura medium (minus casein hydrolysate) containing either no nitrate or with 1, 2 or 4 fold nitrate level.

Incubation : 21 days in light at 26±2°C.

Time (days)	Specific activity* of Enzyme Nitrate concentration (x folds)			
	0	1	2	4
0	Not detectable	Not detectable	Not detectable	Not detectable
3	6.4	9.5	12.8	6.2
6	10.7	14.8	18.3	8.2
9	14.2	20.9	26.8	9.7
12	18.5	28.6	35.4	10.2
15	12.3	25.4	28.2	8.0
18	9.1	15.2	18.6	6.5
21	7.2	10.0	14.0	4.0

Enzyme Units : Amount of enzyme produces 1 µ g of glutamate in 1 hour at 37°C.

*Specific Activity : Number of enzyme units per mg protein.

EXPERIMENT 18 : Effect of different Nitrates in the Medium on Nitrate Reductase and Glutamic Oxaloacetic Transaminase activity in Datura Callus cultures

Tissues growing on Datura medium (Table 4) were transferred to flasks containing a medium lacking nitrate and casein hydrolysate and allowed to grow for 2 weeks. The callus thus grown was used as the inoculum for this experiment. Weighed amounts (100±10 mg) of tissues were transferred to flasks containing $NaNO_3$, KNO_3, $Ca(NO_3)_2$ or NH_4NO_3 as the nitrogen sources, the level of NO_3 ions being kept constant in each case. The cultures were incubated in light at 26±2°C for 21 days. A fixed number of replicates was harvested every third day after inoculation from each treatment. About two grams of tissue was collected from each of the treatments for the enzyme assays (Materials and Methods, 8C,D).

Data presented in Table 34 showed that in all the treatments the activity of the enzyme NR, which was induced during the first 3 days increased upto 12th day and then declined. The values of the enzyme nitrate reductase in the tissues grown on media

Table 34: Effect of different Nitrates in the Medium on Nitrate Reductase activity in *Datura* Callus cultures.

Inoculum : 100±10 mg of tissue in 25 ml of Datura medium (minus casein hydrolysate) containing either
(A) Sodium nitrate,
(B) Potassium nitrate,
(C) Calcium nitrate, or
(D) Ammonium nitrate
(at equimolar concentration).

Incubation : 21 days in light at 26±2°C.

Time (days)	Specific activity* of Enzyme Different Nitrates in the media			
	A	B	C	D
0	Not detectable	Not detectable	Not detectable	Not detectable
3	8.2	8.9	8.0	6.0
6	14.5	15.1	14.7	12.8
9	17.8	17.9	17.8	14.2
12	22.6	22.5	22.9	17.3
15	16.4	16.8	16.0	11.2
18	12.5	13.1	12.8	8.3
21	8.0	9.0	8.5	6.0

Enzyme Units : Amount of enzyme which can reduce 1 mμ mole of substrate in 1 minute

*Specific Activity : Number of enzyme units per mg protein.

Table 35: Effect of Different Nitrates in the Medium on Glutamic Oxaloacetic Transaminase activity in *Datura* Callus cultures

Inoculum : 100±10 mg of tissues in 25 ml of Datura medium (minus casein hydrolysate) containing either
(A) Sodium nitrate,
(B) Potassium nitrate,
(C) Calcium nitrate or
(D) Ammonium nitrate(at equimolar concentration).

Incubation : 21 days in light at 26±2°C.

Time (days)	Specific activity* of Enzyme Different Nitrates in the Media			
	A	B	C	D
0	Not detectable	Not detectable	Not detectable	Not detectable
3	9.5	8.9	9.0	12.6
6	14.8	13.2	14.6	17.2
9	20.9	19.5	21.0	22.8
12	28.6	26.9	29.8	33.6
15	25.4	24.0	26.0	28.2
18	15.2	14.8	14.8	18.3
21	10.0	9.2	9.8	12.5

Enzyme Units : Amount of enzyme produces 1 µ g of glutamate in 1 hour at 37°C.

*Specific Activity : Number of enzyme units per mg protein.

containing $NaNO_3$, KNO_3 or $Ca(NO_3)_2$ as nitrogen sources were more or less equal throughout the course of culture. However, the nitrate reductase activity was consistently less in the tissues grown on medium containing NH_4NO_3.

Progressive changes in the activity of GOT presented in Table 35 showed a different picture. The glutamic oxaloacetic transaminase values remained almost equal in the tissues grown on $NaNO_3$, KNO_3 or $CaNO_3$ containing media. On the other hand, the specific activity of the enzyme was significantly higher throughout the culture period in the medium containing ammonium nitrate as the nitrogen source.

EXPERIMENT 19 : Effect of Casein hydrolysate on Nitrate Reductase and Glutamic Oxaloacetic Transaminase activity in Datura Callus cultures

The callus tissues 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, 150, 300, 600 or 1200 mg/l casein hydrolysate. The cultures were incubated in light at 26±2°C for 21 days. Every third day after inoculation, a fixed number of replicates was harvested from each treatment and tissues collected for the enzyme assays (Materials and Methods, 8C,D).

The progressive changes in the activities of nitrate reductase and glutamic oxaloacetic transaminase in Datura callus grown on media containing various amounts of casein hydrolysate are presented in Table 36. Clearly, casein hydrolysate reduced the nitrate reductase activity and the repression increased with the increase in concentration of casein hydrolysate in the medium. Presence of casein hydrolysate adversely affected the induction of NR during the initial 3 days in culture. On day 12, the peak value of the enzyme attained in tissues grown on media incorporating 1200 mg/l casein hydrolysate, was less than half of that registered in tissues incubated in absence of casein hydrolysate. Growth of Datura cultures was earlier noticed, however, enhanced with increasing concentration of casein hydrolysate in the medium (Experiment 9B, Chapter III).

Table 36: Effect of Casein hydrolysate in the Medium on Nitrate Reductase in *Datura* Callus tissues during the course of Culture

Inoculum : 100±10 mg of tissue in 25 ml of Datura medium (minus casein hydrolysate) supplemented with 0, 150, 300, 600 or 1200 mg/l casein hydrolysate.

Incubation : 21 days in light at 26±2°C.

Time (days)	Specific activity* of Enzyme Casein hydrolysate concentration mg/l				
	0.0	150	300	600	1200
0	Not detectable	Not detectable	Not detectable	Not detectable	Not detectable
3	8.2	7.6	7.5	6.5	4.5
6	14.5	13.2	12.0	10.4	7.0
9	17.8	15.5	14.8	12.2	9.0
12	22.6	20.2	18.5	16.8	10.2
15	16.4	15.2	14.0	12.5	8.0
18	12.5	11.5	10.2	8.9	5.1
21	8.0	7.3	6.5	5.2	3.8

Enzyme Units : Amount of enzyme which can reduce 1 mμ mole of substrate in 1 minute

*Specific Activity : Number of enzyme units per mg protein.

Table 37: Effect of Casein hydrolysate in the Medium on Glutamic Oxaloacetic Transaminase in Datura callus tissue during the course of Culture

Inoculum : 100±10 mg of tissue in 25 ml of Datura medium (minus casein hydrolysate) supplemented with 0, 150, 300, 600 or 1200 mg/l casein hydrolysate.

Incubation : 21 days in light at 26±2°C.

Time (days)	Specific activity* of Enzyme Casein hydrolysate concentration mg/l				
	0.0	150	300	600	1200
0	Not detectable	Not detectable	Not detectable	Not detectable	Not detectable
3	9.5	12.6	13.4	14.2	14.8
6	14.8	16.4	20.3	22.0	28.3
9	20.9	24.5	28.2	36.4	38.5
12	28.6	34.6	39.2	45.0	48.6
15	25.4	27.8	30.2	32.6	36.2
18	15.2	16.6	18.2	24.6	28.0
21	10.2	10.8	12.0	16.2	18.0

Enzyme Units : Amount of enzyme produce 1 µ g of glutamate in 1 hour at 37°C.

*Specific Activity : Number of enzyme units per mg protein.

Fig. 19. Effect of Casein hydrolysate on the Specific activities of Nitrate Reductase (NR) Glutamic Oxaloacetic Transaminase (GOT) in Datura Callus cultures (as observed on 12th day of culture).

100 mg of tissue in 25 ml of Datura medium (either lacking casein hydrolysate or supplemented with 150,300,600 or 1200 mg/l casein hydrolysate)

Other experimental details as given in Tables 36 and 37.

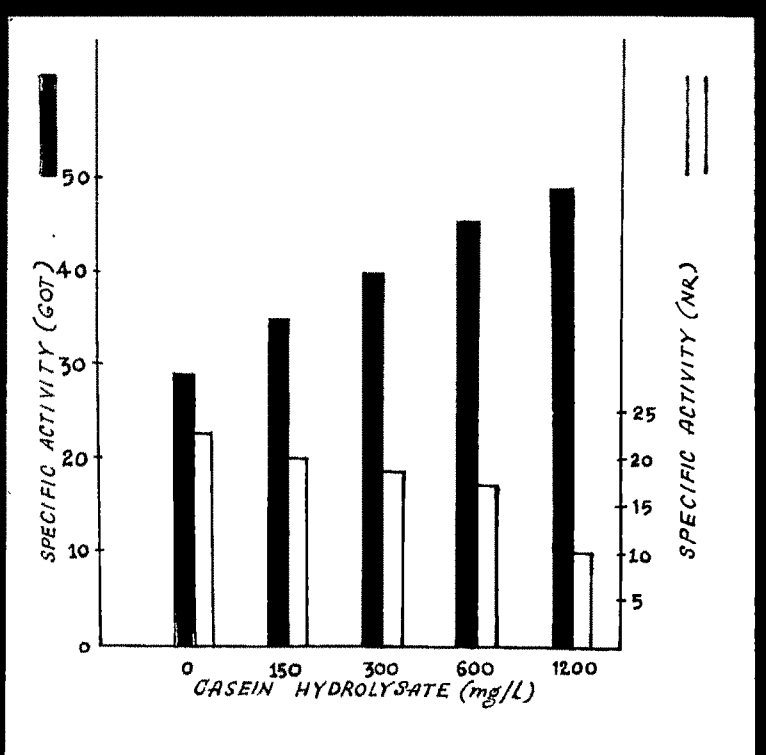
SPECIFIC ACTIVITY (GOT)
0
10
20
30
40
50
SPECIFIC ACTIVITY (NR)
5
10
15
20
25
0
150
300
600
1200
CASEIN HYDROLYSATE (mg/L)

Fig. 19

The specific activity of glutamic oxaloacetic transaminase, on the other hand, was observed (Table 37) to enhance with increasing casein hydrolysate concentration and its pattern simulated the growth attained at various casein hydrolysate concentrations (Experiment 9B, Chapter III). The trends of specific activities of NR and GOT as recorded on 12th day of culture, are illustrated in Figure 19.

EXPERIMENT 20 : Effect of Molybdenum on Nitrate Reductase activity in Datura Callus cultures

Measured amounts of tissues (100±10 mg) were transferred to flasks containing Datura medium (devoid of casein hydrolysate) supplemented with 0, 0.001, 0.01, 0.1 or 1.0 mg/l molybdenum. Prior to inoculation of this experiment, the callus tissues were grown on casein hydrolysate free medium for a period of two weeks to minimise any carry-over effect. The flasks were incubated in light at 26±2°C and a fixed number of replicates was harvested every third day and the callus tissues collected separately from

Table 38: Effect of Molybdenum on Nitrate reductase activity in *Datura* callus cultures

Inoculum : 100±10 mg of tissue in 25 ml of Datura medium (minus casein hydrolysate) supplemented with 0, 0.001, 0.01, 0.1 or 1.0 mg/l molybdenum.

Incubation : 21 days in light at 26±2°C.

Time (days)	Specific activity* of Enzyme Molybdenum concentration mg/l				
	0.0	0.001	0.01	0.1	1.0
0	Not detectable	Not detectable	Not detectable	Not detectable	Not detectable
3	8.2	10.2	7.9	6.0	4.2
6	14.5	16.8	13.8	11.2	9.0
9	17.8	19.2	16.2	14.0	11.8
12	22.6	24.5	20.8	18.2	15.4
15	16.4	18.9	15.0	13.1	10.2
18	12.5	14.8	10.2	8.4	6.2
21	8.0	10.5	7.0	5.6	4.8

Enzyme Units : Amount of enzyme which can reduce 1 mμ mole of substrate in 1 minute.

*Specific Activity : Number of enzyme units per mg protein.

each of the treatments were used for the enzyme assay (Materials and Methods, 8D).

The results presented in Table 38 showed that the specific activity of the enzyme was markedly affected by the presence or absence of molybdenum in the medium. The induction of enzyme as well its activity was relatively higher in tissues grown on media containing 0.001 mg/l molybdenum—the same concentration which supported highest growth of the callus (Experiment 10, Chapter III). The specific activity was suppressed with further increase in concentration of molybdenum, the medium containing 1.0 mg/l molybdenum registering the least activity. The same concentration had earlier been found (Experiment 10, Chapter III) to be toxic for supporting the growth of Datura tissue.

EXPERIMENT 21 : Studies on Acid (β-Glycero) Phosphatase in Datura Callus cultures

Having investigated in the previous experiments the changes in the nitrogen contents and nitrate reductase activity associated with growth of Datura callus, it was felt worthwhile to examine whether the

increase in protein content noted were due to either a general increase in the level of all proteins or due to increase in the levels of only certain proteins. Changes in the pattern of enzyme Acid phosphatase are, therefore, followed in the following experiments. The effect of 2,4-D and kinetin, singly and in combination on the growth of Datura callus has already been studied (Experiments 2 and 6, Chapter III).

A. Effect of Auxin on Acid Phosphatase activity :

To study the effect of auxin (2,4-D) on acid phosphatase activity, tissue pieces of Datura callus weighing 100±10 mg each were inoculated separately in Erlenmeyer flasks containing 25 ml of Datura medium supplemented with either 0, 0.2, 2.0 or 20.0 mg/l 2,4-D. Prior to the inoculation of the experiment, the callus tissues were grown on auxin free medium for a period of two weeks to minimise carry-over effect. The flasks were incubated in light at a constant temperature of 26±2°C. A fixed number of replicates was harvested on every fifth day after inoculation and about two grams of tissues were collected from each treatment for enzyme extraction and assay. The methods employed for enzyme assay are

Table 39: Effect of 2,4-D on Acid Phosphatase in *Datura* Callus cultures

Inoculum : 100±10 mg of tissue in 25 ml of Datura medium (minus 2,4-D) supplemented with 0.0, 0.2, 2.0 or 20.0 mg/l 2,4-D.

Incubation : 25 days in light at 26±2°C.

Time (days)	2,4-D (mg/l)	Specific activity* of Enzyme
0	0.0	41
5	0.0	49
	0.2	67
	2.0	75
	20.0	35
10	0.0	69
	0.2	83
	2.0	107
	20.0	72
15	0.0	77
	0.2	105
	2.0	135
	20.0	75
20	0.0	86
	0.2	121
	2.0	137
	20.0	91
25	0.0	47
	0.2	82
	2.0	100
	20.0	45

Enzyme Units : Amount of enzyme liberating 1 µg of inorganic phosphate from substrate in 90 minutes at 37°C.

*Specific Activity : Number of enzyme unit per mg protein.

Fig. 20. Effect of 2,4-D on Acid phosphatase in Datura Callus cultures.

100 mg of tissue in 25 ml of Datura medium (minus 2,4-D) supplemented with 0, 0.2, 2.0 or 20.0 mg/l 2,4-D.

Other experimental details as given in Table 39.

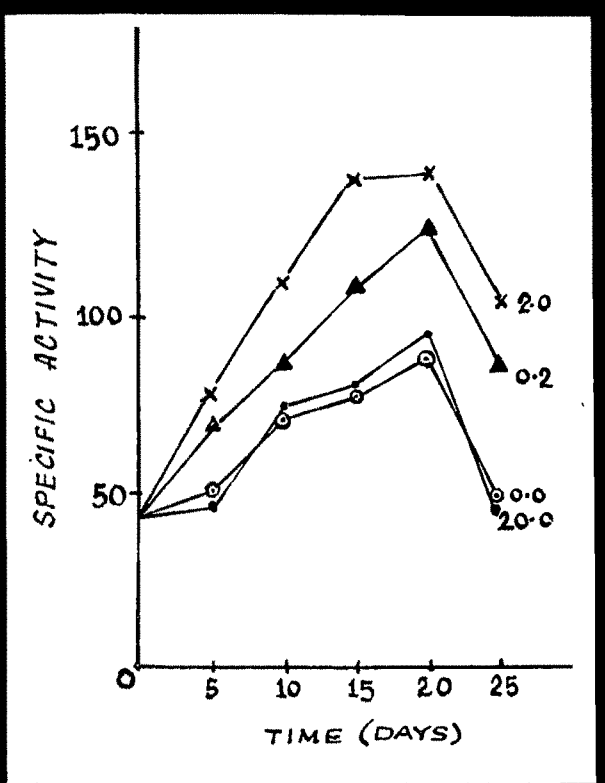
150
100
50
0
SPECIFIC ACTIVITY
5
10
15
20
25
TIME (DAYS)
2·0
0·2
0·0
20·0

Fig. 20

described in Materials and Methods, 8A.

The results obtained are presented in Table 39 and Figure 20. Clearly, the specific activity of the enzyme acid phosphatase rose during the course of culture in all the treatments including the control; the peak values being achieved on 20th day of inoculation after which the activity declined. The enzyme activity was consistantly and appreciably higher in medium containing 2.0 mg/l 2,4-D—the auxin concentration which had been earlier found optimal for growth of the tissues (Experiment 2, Chapter III). Acid phosphatase activity in presence of higher level of 2,4-D was nearly as low as that in the total absence of it.

B. Effect of Kinetin on Acid phosphatase in Datura Callus cultures:

Experiment was next conducted to study the effect of kinetin on acid phosphatase activity. Tissues growing on Datura medium were transferred on to medium devoid of auxin and incubated for two weeks. 100±10 mg of tissues from above were then inoculated separately in the flasks containing 25 ml of Datura medium

lacking auxin but supplemented with 0.0, 0.05, 0.5 or 5.0 mg/l kinetin. The flasks were incubated in light at 26±2°C. A fixed number of replicates was harvested on every fifth day after inoculation. About two grams of tissues were collected from each of the treatments for enzyme assay (Materials and Methods, 8A).

The results presented in Table 40 and Figure 21 showed that there was a considerable increase in the specific activity of the enzyme acid phosphatase during the course of culture in all the treatments including the control. The maximum activity was recorded on 20th day after inoculation of the experiment and this was followed by general decline in all the treatments. The highest activity of the enzyme was achieved at 0.5 mg/l kinetin concentration which also supported the maximum growth of the tissue in culture (Experiment 6, Chapter III). High kinetin (5.0 mg/l) level in the medium repressed the enhancement in enzyme activity though the activity was never as low here as in complete absence of kinetin.

Table 40: Effect of Kinetin on Acid Phosphatase in *Datura* Callus cultures

Inoculum : 100±10 mg of tissue in 25 ml of Datura medium (minus 2,4-D) supplemented with 0.0, 0.05, 0.5 or 5.0 mg/l kinetin.

Incubation : 25 days in light at 26±2°C.

Time (days)	Kinetin (mg/l)	Specific activity* of Enzyme
0	0.00	40
5	0.00	50
	0.05	72
	0.50	85
	5.00	56
10	0.00	69
	0.05	91
	0.50	117
	5.00	77
15	0.00	78
	0.05	112
	0.50	149
	5.00	86
20	0.00	88
	0.05	131
	0.50	187
	5.00	93
25	0.00	49
	0.05	83
	0.50	124
	5.0o	64

Enzyme Units : Amount of enzyme liberating 1 µ g of inorganic phosphate from substrate in 90 minutes at 37°C.

*Specific Activity : Number of enzyme units per mg protein.

Fig. 21. Effect of Kinetin on Acid phosphatase in *Datura* Callus cultures.

100 mg of tissue in 25 ml of Datura medium (2,4-D) supplemented with 0, 0.05, 0.5 or 5.0 mg/l kinetin.

Other experimental details as given in Table 40.

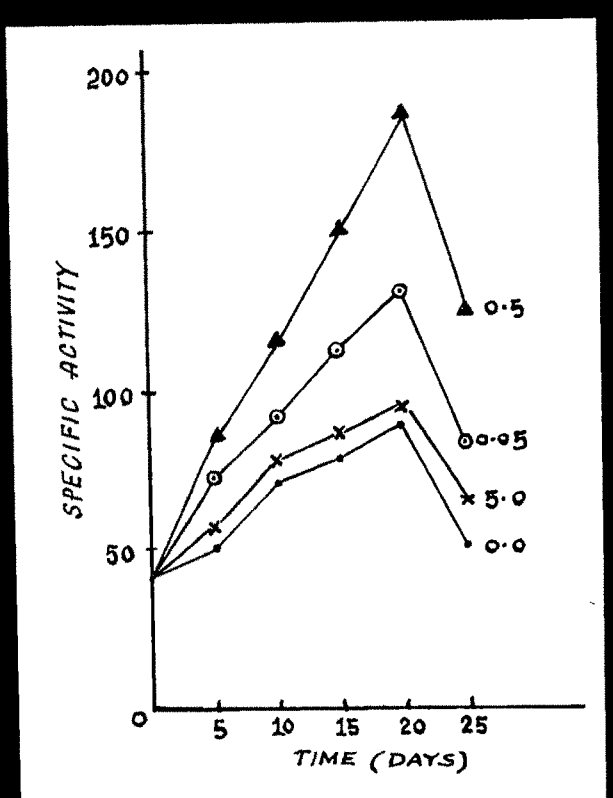
200
150
100
50
0
SPECIFIC ACTIVITY
5
10
15
20
25
TIME (DAYS)
0·5
0·05
5·0
0·0

Fig. 21

C. Effect of Kinetin in presence of Auxin on Acid phosphatase Activity in Datura Callus cultures:

To study the combined effect of auxin and kinetin on acid phosphatase activity, 100±10 mg of tissue pieces were transferred separately to flasks containing 25 ml of Datura medium (Table 4) supplemented with 0.0, 0.05, 0.5 or 5.0 mg/l kinetin. The cultures were incubated in light at a constant temperature of 26±2°C. A fixed number of replicates was harvested on every 5th day after inoculation. About two grams of tissues were collected from each of the treatments at every harvest for the enzyme assay (Materials and Methods, 8A).

The results presented in Table 41 revealed that as in previous experiments the activity of the enzyme increased with the age of the culture upto 20th day. With optimal level of auxin (2.0 mg/l 2,4-D) in the medium the enzyme achieved maximum activity on 20th day in presence of 0.5 mg kinetin. It was the same concentration of kinetin which was earlier observed to support highest growth (Experiment 6, Chapter III) as well as enzyme activity (Experiment 21B, Chapter IV). At this combination of 2,4-D (2.0 mg/l) and kinetin (0.5 mg/l) the specific activity

of the enzyme was considerably more as compared to the activity recorded on medium containing either 2,4-D or kinetin alone. It was further observed that in presence of 2,4-D (2.0 mg/l) the difference in the activity of acid phosphatase in tissues grown on 0.05 and 0.5 mg/l kinetin media was much reduced when compared with the values at the same kinetin concentrations in absence of 2,4-D noted in earlier part (Experiment 21B) of this experiment.

Table 41: Effect of Auxin and Kinetin on Acid Phosphatase in *Datura* Callus cultures

Inoculum : 100±10 mg of tissue in 25 ml of Datura medium supplemented with 0.0, 0.05, 0.5 or 5.0 mg/l kinetin.

Incubation : 25 days in light at 26±2°C.

Time (days)	Kinetin (mg/l)	Specific activity* of Enzyme
0	0.00	41
5	0.00	79
	0.05	84
	0.50	92
	5.00	76
10	0.00	115
	0.05	124
	0.50	128
	5.00	113
15	0.00	138
	0.05	161
	0.50	165
	5.00	140
20	0.00	179
	0.05	189
	0.50	197
	5.00	147
25	0.00	101
	0.05	117
	0.50	128
	5.00	100

Enzyme Units : Amount of enzyme liberating 1 µg of inorganic phosphate from substrate in 90 minutes at 37°C.

*Specific Activity : Number of enzyme units per mg protein.

Fig. 22. Changes in the Nitrate Reductase (NR) and Acid Phosphatase activities in *Datura* Callus during the course of culture.

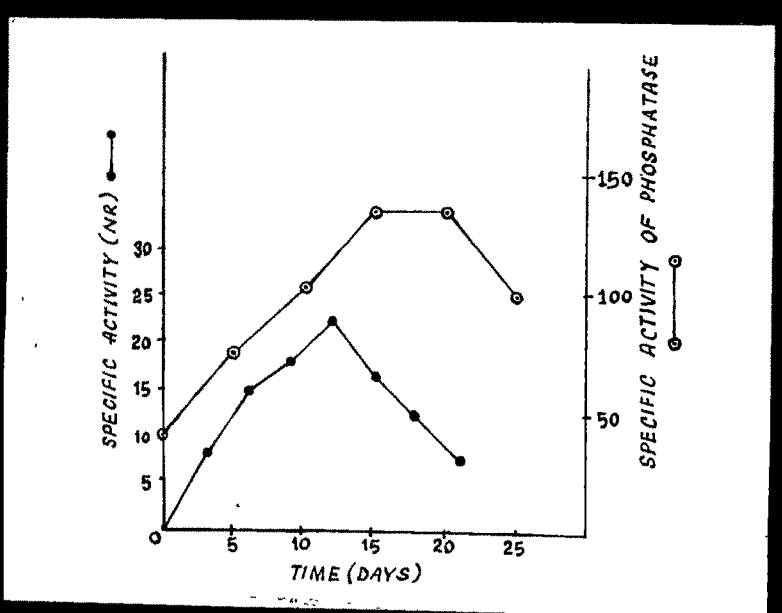
SPECIFIC ACTIVITY (NR)
30
25
20
15
10
5
0
5
10
15
20
25
TIME (DAYS)
150
100
50
SPECIFIC ACTIVITY OF PHOSPHATASE

Fig. 22

DISCUSSION

Callus tissues grown on sucrose media contained higher amounts of total and bound sugars than free sugars; while the tissues grown on other carbohydrates including equimolar mixture of glucose and fructose had more of free sugars than bound ones. The activity of invertase was also higher in tissues grown on sucrose medium than those cultured on glucose or fructose media; the peak value as recorded on day 15 being twice as high in case of sucrose medium (Experiment 11). Furthermore, the ratio of bound sugars to free sugars as well as the invertase activity was found to enhance with increasing level of sucrose in the medium (Experiment 12).

Growth as measured by increase in dry weight of the tissues cultured on different carbon sources was highest on sucrose containing medium and the lowest in tissues on medium containing soluble starch. The growth on latter, however, was appreciably more than that in complete absence of carbon source (Experiment 11). Growth of the tissue enhanced with increase in sucrose concentration upto 2 per cent; but the dry

weight per cell continued to enhance with increasing sucrose concentration upto 4 per cent (Experiment 12). These findings seemed to suggest that the greater amount of bound sugar than free sugar in tissues accounts for superiority of sucrose over other carbohydrates tested. The corelation between growth and invertase activity is discussed at length in General Discussion (Chapter V).

When the changes in the nitrogen content in callus cultures were examined, there was noticed a sharp rise in cellular nitrogen per unit dry weight as well as per cell basis during the initial period of culture, when there were no comparable increases in dry weight or total cell number. When the nitrogen fractions declined after attaining the peak values on day 12, the growth enhanced rapidly (Experiment 13).

Examination of nitrogen contents in Datura callus tissues grown on sucrose or glucose containing media showed that total nitrogen content was significantly higher in sucrose grown tissues than the glucose grown ones (Experiment 14). The glutamic oxaloacetic transaminase activity aslo revealed similar picture (Experiment 15).

The examination of nitrogen requirement in callus cultures was pursued further by studying the changes in nitrate reductase and glutamic oxaloacetic transaminase activities and the effects of nitrogen level and different sources of nitrogen on the patterns of their activities during the course of culture. Both the enzymes were induced in first 3 days of culture and developed peak activities on day 12 before declining (Experiment 16). With the increase in level of nitrate in the medium, the nitrate reductase activity enhanced. The glutamic oxaloacetic transaminase activity, however, enhanced upto double the level of nitrate in the medium; while in tissues grown on medium containing 4 fold nitrate supply the enzyme activity was even less than that registered in the medium which lacked nitrate (Experiment 17). Thus the glutamic oxaloacetic transaminase pattern simulated growth attained on different nitrate levels (Experiment 8).

The nitrate reductase activity in tissues cultured on media containing either sodium nitrate, potassium nitrate or calcium nitrate as nitrogen sources was nearly equal. In presence of ammonium nitrate as the

nitrogen source the activity was, however, consistently low. The progress of glutamic oxaloacetic transaminase activity showed a different pattern. While it remained more or less equal in tissues grown on sodium nitrate, potassium nitrate or calcium nitrate containing media, it registered appreciable enhancement in ammonium nitrate incubated tissues (Experiment 18). Clearly like nitrate level, the nitrate compounds also had similar effect on growth (Experiment 9) and glutamic oxaloacetic transaminase activity in the tissues.

Addition of casein hydrolysate in the nitrogen containing medium reduced the nitrate reductase activity, the repression becoming more pronounced with the increasing concentration of casein hydrolysate in the medium. In contrast, the glutamic oxaloacetic transaminase activity, like growth (Experiment 9B), enhanced with increasing level of casein hydrolysate (Experiment 19). Studies with the effect of molybdenum on Datura callus cultures (Experiment 20) showed that the enzyme activity was enhanced in presence of its minute dose (0.001 mg/l), the same concentration being earlier found optimal for tissue growth (Experiment 10).

Both auxin and kinetin, singly and in combination, caused pronounced enhancement in the activity of acid phosphatase (Experiment 21). At their optimal concentrations, kinetin had greater stimulatory effect than 2,4-D. The patterns of nitrate reductase and acid phosphatase activities were similar during the first 12 days. The nitrate reductase activity declining a little earlier and faster than that of acid phosphatase.