CHAPTER V

STUDIES ON PEROXIDASE, INDOLEACETIC ACID OXIDASE AND PHENYLALANINE AMMONIA-LYASE (PAL) ENZYMES IN RELATION TO POLYPHENOL SYNTHESIS

STUDIES ON PEROXIDASE, INDOLEACETIC ACID OXIDASE AND PHENYLALANINE AMMONIA-LYASE (PAL) ENZYMES IN RELATION TO POLYPHENOL SYNTHESIS

Substances like carbohydrates, auxin and kinetin regulated growth and polyphenol synthesis in cultured plant tissues as was realised in earlier experiments presented in Chapter IV. Further, these substances were also found by several workers to affect important enzyme systems such as peroxidase, indoleacetic acid oxidase (IAA oxidase) and others which play important role in the physiology of growth. Also, the peroxidase was found to be an essential part of IAA oxidase system, as it was known to catalize the oxidation of IAA (Galston and McCune, 1961; McCune, 1961). Furthermore, it was demonstrated that peroxidase played a role in several reactions related to phenolic biosynthesis (Mason et al., 1957).

Yet another key enzyme, phenylalanine ammonia-lyase (PAL) which was responsible for the deamination of the aromatic amino acid, phenylalanine, leading to the formation of phenylpropanoid compounds, was also found to be influenced by several factors such as carbohydrates, hormones and light (Creasy, 1968; Cheng and Marsh, 1968; Ruberye and Fosket, 1969; Davies, 1972; Zucker, 1965).

Experiments described in the present chapter were, therefore, planned with the objective to examine the effect of sucrose, 2,4-D and kinetin on peroxidase, IAA oxidase and PAL enzymes in relation to polyphenol synthesis and growth of Datura and Cassia tissue cultures.

As mentioned in the introduction, there are reports on the release of enzymes like peroxidase, IAA oxidase and others into the culture Medium. However, no detailed studies on their release as a function of time and as influenced by cultural procedures, nor on the effect of these released enzymes on growth and metabolism of cultured plant cells are available. In the present investigations attempts were, therefore, made to examine any correlation between peroxidase and IAA oxidase enzymes as detected in the tissues and in the media and their influence on growth and polyphenol production, if any.

Experiment 5-1 : Effect of Sucrose Concentrations on Peroxidase and Indoleacetic acid Oxidase (IAA oxidase) Activities in Suspension Cultures of Datura

Measured aliquots of regularly subcultured suspension, weighing approximately 300±30 mg tissue by fresh weight, were tfansferred to 40 ml of modified MS medium (Table 3, Chapter II) supplemented with 0.0, 2.0 or 4.0% sucrose in addition to 2.0 mg/l 2,4-D and 0.4 mg/l kinetin. The flasks were continuously agitated on a shaker in light in a constant temperature (26±2°C) culture room. A fixed number of replicates was harvested at five days interval upto 20 days. The tissues from each treatment were pooled separately and assayed for peroxidase and IAA oxidase enzyme activities as described in Materials and Methods, 7A and B (Chapter II).

The peroxidase activity expressed as the rate of increase in optical density/minute at 470 mμ and presented in Table 21 and Fig. 29 showed a slight change during initial 5 days of culture. The activity, however, increased rapidly during 5 to 10 days and continued to raise in the subsequent period of 10 to 15 days in cells grown in sucrose

medium. In absence of sucrose, on the other hand, the activity was less pronounced and its peak activity was also attained much earlier. Further, in the cells incubated in sucrose medium, the enzyme activity dropped sharply after 15 days, whereas in sucrose free medium the fall was gradual after day 10.

The rapid increase in peroxidase activity during the period 5 to 15 days corresponded both with high polyphenol synthesis (taking place during 5 to 10 days) and rapid growth which occurred during 10 to 15 days as already described in Experiment 4-2.

Unlike peroxidase, the IAA oxidase activity showed a steep rise during the initial five days (Fig. 30) which corresponded to the lag phase in growth (Experiment 3-2). It continued to raise in pre-exponential growth phase (5 to 10 days) in all the treatments reaching peak activity on day 10; it was during the same 5-10 day period that maximum polyphenol synthesis was observed (Experiment 4-2). The activity declined after day 10 more sharply in presence of sucrose than in its absence. As in the case of peroxidase, the maximum IAA oxidase activity was registered in higher sucrose level (4%) which supported maximum polyphenol synthesis rather than in 2% sucrose

Table 21 : Effect of Sucrose Concentrations on Peroxidase Activity in *Datura* Cell Suspension Cultures

Inoculum : 300±30 mg tissue by fresh weight in 40 ml of modified MS medium (Table 3, Chapter II) supplemented with 0.0, 2.0 or 4.0% sucrose.

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

Time (days)	Sucrose (%)	Peroxidase activity* (OD)
0	0	0.035
5	0	0.060
	2	0.040
	4	0.075
10	0	0.160
	2	0.210
	4	0.180
15	0	0.110
	2	0.370
	4	0.415
20	0	0.030
	2	0.055
	4	0.270

*Peroxidase activity: It is expressed as the rate of increase in OD/min at 470 mμ.

Table 22 : Effect of Sucrose Concentrations on IAA Oxidase Activity in *Datura* Cell Suspension Cultures

Inoculum : 300±30 mg tissue by fresh weight in 40 ml of modified MS medium (Table 3, Chapter II) supplemented with 0.0, 2.0 or 4.0% sucrose.

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

Time (days)	Sucrose (%)	IAA oxidase activity* (µg)	Specific activity**
0	0	80	1.200
5	0	145	8.834
	2	175	12.500
	4	190	16.412
10	0	150	14.023
	2	310	26.312
	4	380	32.421
15	0	115	10.124
	2	190	12.212
	4	230	26.400
20	0	85	2.000
	2	235	10.112
	4	200	8.302

*IAA oxidase activity: µg of IAA destroyed/mg dry weight of the tissue in 25 min.

**Specific activity: µg of IAA destroyed/µg protein.

Fig. 29. Changes in peroxidase activity of Datura cell suspensions grown in different sucrose concentrations.

Inoculum size: 300±30 mg tissue in 40 ml of modified MS medium supplemented with 0.0, 2.0 or 4.0% sucrose.

Experimental details as given in Table 21.

Fig. 30. Changes in IAA oxidase activity of Datura cell suspensions grown in different sucrose concentrations.

Inoculum size: 300±30 mg tissue in 40 ml of MS medium supplemented with 0.0, 2.0 or 4.0% sucrose.

Experimental details as given in Table 22.

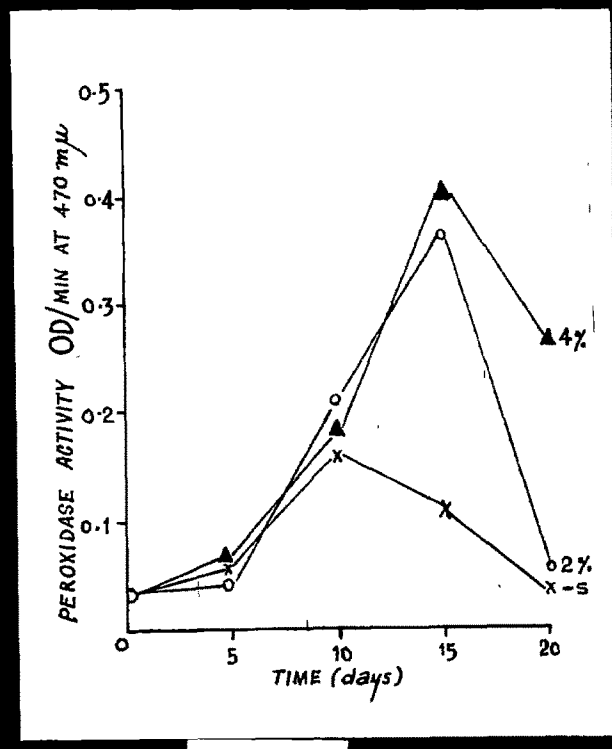
PEROXIDASE ACTIVITY OD/MIN AT 470 mμ
0·5
0·4
0·3
0·2
0·1
0
5
10
15
20
TIME (days)
4%
2%
x-s

Fig. 29

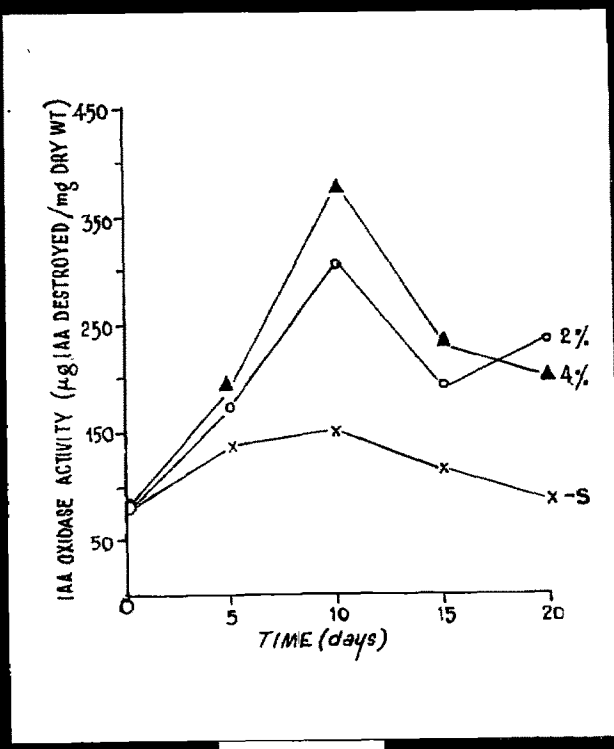
IAA OXIDASE ACTIVITY (μg IAA DESTROYED/mg DRY WT)
450
350
250
150
50
0
5
10
15
20
TIME (days)
2%
4%
x-S

Fig. 30

which was earlier found to be optimal for growth (Experiment 4-2). There was further enhancement of IAA oxidase activity in 2% sucrose after day 15.

Experiment 5-2 : Release of Peroxidase and Indoleacetic acid Oxidase Enzymes into the Culture Medium as Influenced by Sucrose levels

Measured aliquots of regularly subcultured cell suspension weighing approximately 300±30 mg by fresh weight, were inoculated into 150 ml capacity Erlenmeyer flasks containing 40 ml of modified MS medium (Table 3, Chapter II), supplemented with 0.0, 2.0 or 4% sucrose, in addition to 2.0 mg/l 2,4-D and 0.4 mg/l kinetin. The flasks were continuously agitated on a rotary shaker in light at a constant temperature of 26±2°C.

A fixed number of flasks was harvested at 5 days interval upto 20 days. The culture medium of each treatment was separated from the tissue by filtering through sintered glass Gooch crucible under pressure. The separated medium was then centrifuged at 10,000 x g for 20 minutes and then it was dialyzed against 4 litres of distilled water with 2 to 3 changes at 4 hours interval. All the operations were carried out at 2 to 4°C. The dialyzed medium was again

centrifuged at 5000 x g for 10 minutes at 2°C and the supernatant thus obtained was used as enzyme source. Peroxidase and IAA oxidase activities were estimated in aliquots of the enzyme, according to the standard procedures described in Chapter II, Materials and Methods, 7A and B.

The results presented in Table 23 and illustrated in Fig. 31 and 32 clearly indicated that large amounts of peroxidase and IAA oxidase enzymes were released into the medium during the course of growth in culture.

Peroxidase secretion by Datura cells into the medium was considerably high as compared to IAA oxidase secretion. Further, without any initial delay, both the enzymes have leached out into the medium even in the lag phase of growth. Peroxidase was secreted in greater amounts into the medium containing 4% sucrose, as was observed within the tissues as well (Experment 5-1). The cells grown in medium without sucrose also released both the enzymes at all stages of growth. The maximum secretion of peroxidase and IAA oxidase was observed on day 15 and 10 respectively, as in the case of the cells (Experiment 5-1). Furthermore, as it was noticed in tissues, a rise in IAA oxidase activity was observed after day 15.

Table 23 : Effect of Sucrose on the Release of Peroxidase and IAA Oxidase Enzymes into the Culture Medium

Inoculum : 300±30 mg tissue by fresh weight in 40 ml of modified MS medium (Table 3, Chapter II) supplemented with 0.0, 2.0 or 4.0% sucrose.

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

Time (days)	Sucrose (%)	IAA oxidase activity* (µg)	Peroxidase activity** (OD)
0	-	-	-
5	0	125	0.200
	2	90	0.460
	4	110	0.330
10	0	160	0.280
	2	280	0.500
	4	415	0.460
15	0	165	0.450
	2	210	0.880
	4	280	1.000
20	0	150	0.430
	2	260	0.780
	4	230	0.940

*IAA Oxidase activity: µg of IAA destroyed/mg dry weight of the tissue in 25 min.

**Peroxidase activity: It is expressed as the rate of increase in OD/min at 470 mµ.

Fig. 31 & 32. Changes in the activity of peroxidase (Fig. 31) and IAA oxidase (Fig. 32) enzymes released into the culture medium of *Datura* cell suspensions as influenced by sucrose levels.

Inoculum size: 300±30 mg tissue in 40 ml of modified MS medium supplemented with 0.0, 2.0 or 4.0% sucrose.

Experimental details as given in Table 23.

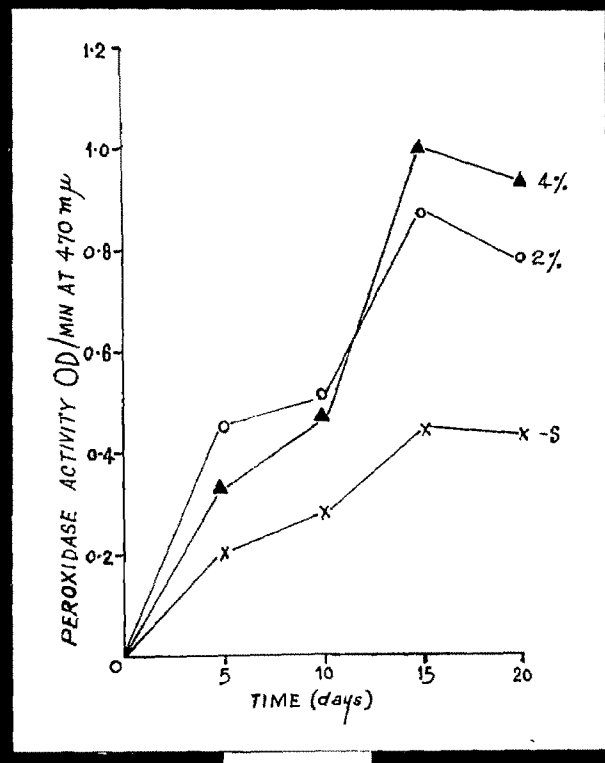
PEROXIDASE ACTIVITY OD/MIN AT 470 mμ
1·2
1·0
0·8
0·6
0·4
0·2
0
5
10
15
20
TIME (days)
4%
2%
-S

Fig. 31

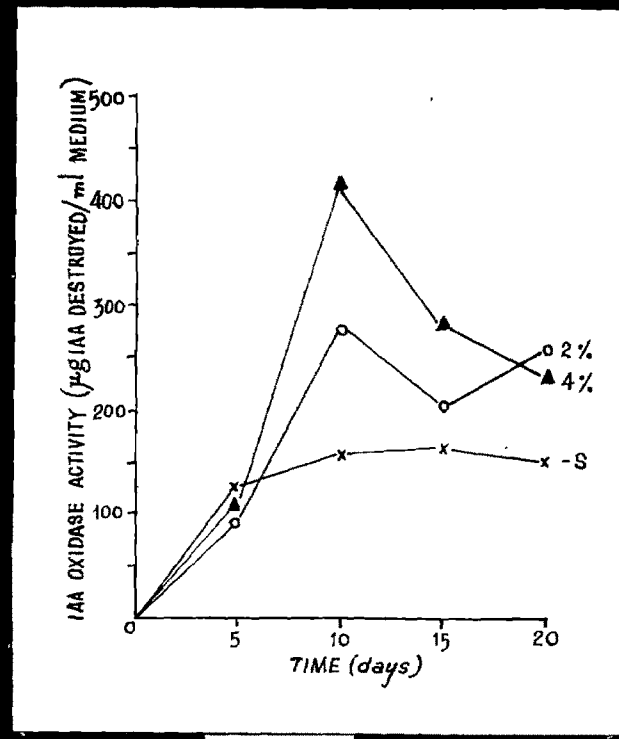
IAA OXIDASE ACTIVITY (μg IAA DESTROYED/ml MEDIUM)
500
400
300
200
100
0
5
10
15
20
TIME (days)
2%
4%
-S

Fig. 32

Clearly, the pattern of changes in IAA oxidase activity was very similar both in the tissues and in the medium. In the case of peroxidase, however, the picture was different particularly during the first 10 days.

Experiment 5-3 : Changes in Peroxidase and Indoleacetic acid Oxidase Activities in Relation to Polyphenol Synthesis as Influenced by 2,4-D Levels in Datura Cell Cultures

Measured aliquots of cell suspension weighing approximately 300±30 mg by fresh weight were transferred to flasks containing 40 ml of modified MS medium (Table 3, Chapter II), supplemented with 0, 10^{-6}, 10^{-5}, $2x10^{-5}$ or $5x10^{-5}$M 2,4-D in addition to 0.4 mg/l kinetin and 2% sucrose. The culture flasks were incubated under identical conditions as stated earlier. Replicate number of flasks was harvested at 5 days interval upto 20 days. The tissues of each treatment were pooled separately for assaying peroxidase and IAA oxidase enzyme activities as described in Chapter II, Materials and Methods, 7A and B.

The results presented in Table 24 and Fig. 33 clearly indicated that the initial effect of 2,4-D was to inhibit

the formation of peroxidase during lag phase of growth at all concentrations. However, at 10^{-5}M and $2x10^{-5}$M 2,4-D concentrations, the activity though slow in the beginning, a remarkable raise in activity was observed upto day 15 and thereafter it declined. The maximum increase in growth observed at 10^{-5}M 2,4-D level might be due to the greater destruction of endogenous auxin by higher peroxidase activity also registered at that concentration. In tissues grown on medium without 2,4-D an initial raise upto day 5 followed by a gradual decline in activity was registered; whereas in other 2,4-D treatments, the peak in activity was observed on day 15.

Contrary to peroxidase formation, an initial sharp raise in IAA oxidase activity was observed upto day 5 at all levels of 2,4-D tested (Fig. 34), indicating the stimulatory effects of 2,4-D on IAA oxidase during the early stages of cell growth. The rate of activity increased furtner and reached its peak on day 10 and thereafter it declined. The very high concentration of 2,4-D ($5x10^{-5}$M) was found to be inhibitory on the enzyme activity as it declined from day 5 onwards. Further, in tissues grown in absence of 2,4-D the activity declined steadily after day 5, indicating the importance of auxin on IAA oxidase activity.

Table 24 : Effect of 2,4-D Concentrations on Peroxidase Activity in Datura Cell Suspension Cultures

Inoculum : 300±30 mg tissue by fresh weight in 40 ml of modified MS medium (Table 3, Chapter II) supplemented with 0, 10^{-6}, 10^{-5}, $2x10^{-5}$ or 5×10^{-5}M 2,4-D.

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

Time (days)	2,4-D (M)	Peroxidase activity* (OD)
0	0	0.035
5	0	0.110
	10^{-6}	0.020
	10^{-5}	0.061
	$2x10^{-5}$	0.060
	$5x10^{-5}$	0.030
10	0	0.075
	10^{-6}	0.160
	10^{-5}	0.375
	$2x10^{-5}$	0.340
	$5x10^{-5}$	0.175
15	0	0.025
	10^{-6}	0.200
	10^{-5}	0.625
	$2x10^{-5}$	0.430
	$5x10^{-5}$	0.335
20	0	0.015
	10^{-6}	0.025
	10^{-5}	0.180
	$2x10^{-5}$	0.125
	$5x10^{-5}$	0.050

*Peroxidase activity: It is expressed as the rate of increase in OD/min at 470 mμ.

Table 25 : Effect of 2,4-D Concentrations on IAA Oxidase Activity in Datura Cell Suspension Cultures

Inoculum : 300±30 mg tissue by fresh weight in 40 ml of modified MS medium (Table 3, Chapter II) supplemented with 0, 10^{-6}, 10^{-5}, $2x10^{-5}$ or $5x10^{-5}$M 2,4-D.

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

Time (days)	2,4-D (M)	IAA Oxidase activity* (µg)	Specific activity**
0	0	83	1.213
5	0	125	6.312
	10^{-6}	226	9.002
	10^{-5}	240	11.426
	$2x10^{-5}$	170	12.506
	$5x10^{-5}$	210	10.478
10	0	105	8.320
	10^{-6}	330	21.241
	10^{-5}	410	32.640
	$2x10^{-5}$	300	22.468
	$5x10^{-5}$	130	8.200
15	0	60	4.120
	10^{-6}	155	8.614
	10^{-5}	325	26.216
	$2x10^{-5}$	195	16.200
	$5x10^{-5}$	75	2.360
20	0	35	1.250
	10^{-6}	250	20.216
	10^{-5}	360	20.150
	$2x10^{-5}$	230	18.680
	$5x10^{-5}$	60	1.840

*IAA Oxidase activity: µg of IAA destroyed/mg dry weight of the tissue in 25 min.

**Specific activity: µg of IAA destroyed/µg protein.

Fig. 33 & 34. Changes in peroxidase (Fig. 33) and IAA oxidase (Fig. 34) activities of *Datura* cell suspensions grown in different 2,4-D concentrations.

Inoculum size: 300±30 mg tissue in 40 ml of modified MS medium supplemented with 0, 10^{-6}, 10^{-5}, $2x10^{-5}$ or $5x10^{-5}$M 2,4-D.

Experimental details as given in Tables 24 and 25.

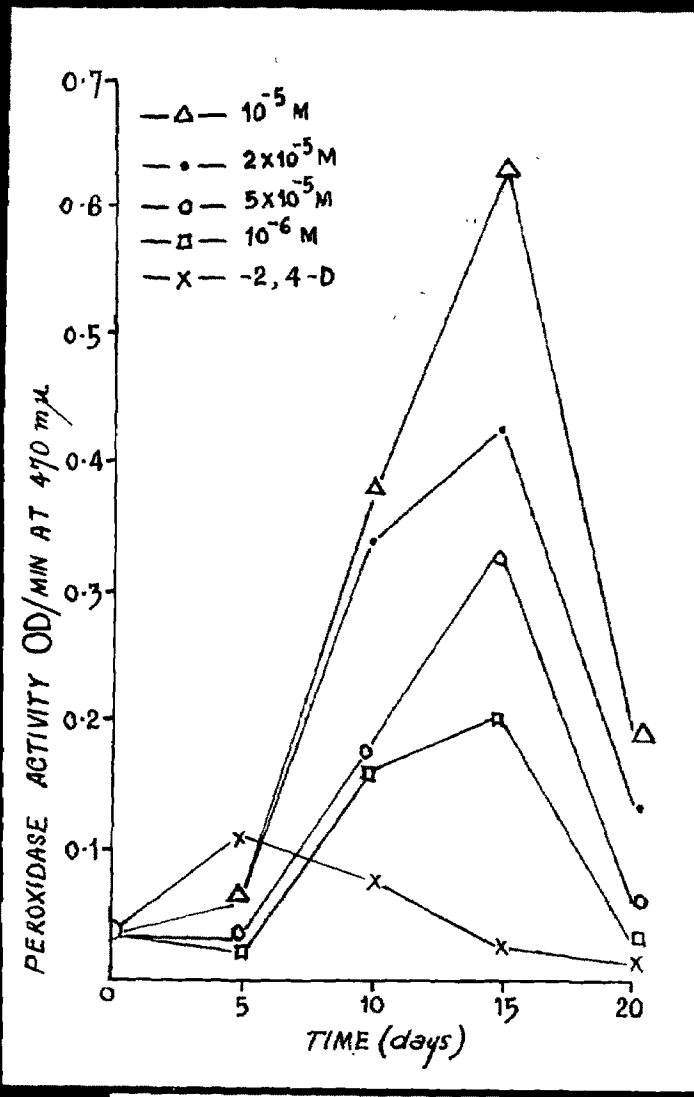
0·7
0·6
0·5
0·4
0·3
0·2
0·1
0
5
10
15
20
—Δ— 10⁻⁵ M
—•— 2x10⁻⁵M
—o— 5x10⁻⁵M
—□— 10⁻⁶ M
—x— -2,4-D
PEROXIDASE ACTIVITY OD/MIN AT 470 mμ
TIME (days)

Fig. 33

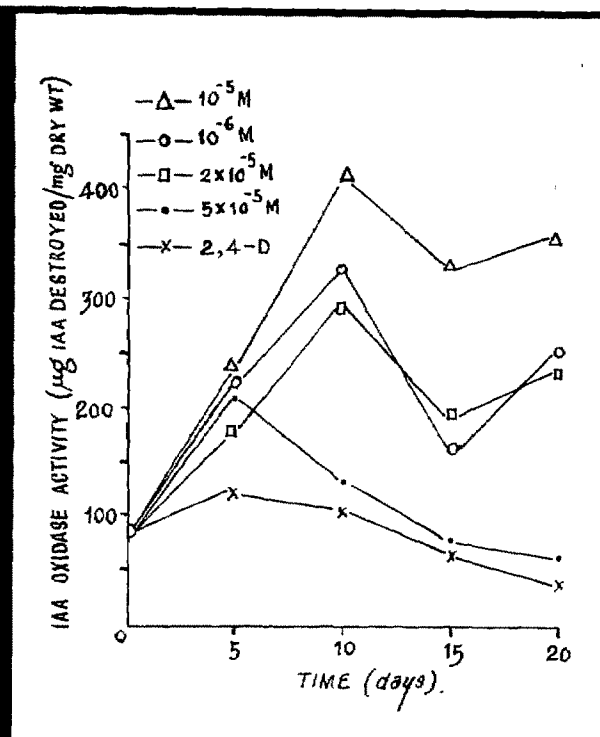
—Δ— 10⁻⁵M
—o— 10⁻⁶M
—□— 2x10⁻⁵M
—•— 5x10⁻⁵M
—x— 2,4-D
400
300
200
100
0
5
10
15
20
IAA OXIDASE ACTIVITY (μg IAA DESTROYED/mg DRY WT)
TIME (days)

Fig. 34

Though the rate of enzyme activity declined on day 15 in cells grown on 10^{-6}, 10^{-5} and $2x10^{-5}$M 2,4-D concentrations, a further raise in activity has been registered after day 15 i.e. in the stationary phase of growth.

The maximum activity of both the enzymes was noted at 10^{-5}M 2,4-D level which also supported maximum growth and polyphenol production in the tissue.

Experiment 5-4 : Effect of 2,4-D on the Release of Peroxidase and Indoleacetic Acid oxidase enzymes into the Culture Medium

40 ml of modified MS medium (Table 3, Chapter II) supplemented with 10^{-5} or $2x10^{-5}$M 2,4-D in addition to 2% sucrose and 0.4 mg/l kinetin was distributed in Erlenmeyer flasks. Measured aliquots of cell suspension weighing approximately 300±30 mg by fresh weight was inoculated into each flask and the culture flasks were incubated under similar conditions as mentioned in Experiment 5-2. Replicate number of flasks was harvested at 5 days interval upto 20 days for the determination of peroxidase and IAA oxidase activities in the spent medium. The spent medium collected at intervals of time was centrifuged and dialyzed

for obtaining enzyme source as described earlier (Experiment 5-2). The enzyme activities were assayed according to standard procedures described in Chapter II, Materials and Methods 7A and B.

The results presented in Table 26 and illustrated in Fig. 35 revealed that very high amounts of peroxidase secretion was found at $2x10^{-5}$M 2,4-D containing medium over control (10^{-5}M). The rate of peroxidase activity was sharp and high in both lag (0 to 5 days) and exponential phase (10-15 days) of the growth and the peak of the activity was recorded on day 15, before declining. This kind of secretion of high amounts of peroxidase at supra-optimal level of auxin, might cause inhibitory effects on the further growth of the cells.

The IAA oxidase secretion into the medium was also observed upto day 10 and thereafter it declined (Fig. 36, Table 26). Though maximum amounts of secretion of the enzyme was observed at $2x10^{-5}$M 2,4-D level, it was not as much as that of peroxidase; probably greater amount of enzyme must have been retained in the tissue. This also seemed to reflect on the binding capacity of the enzyme protein to the cell wall.

Table 26 : Effect of 2,4-D Concentrations on the Release of Peroxidase and IAA Oxidase Enzymes into the Culture Medium

Inoculum : 300±30 mg tissue by fresh weight in 40 ml of modified MS medium (Table 3, Chapter II) supplemented with 10^{-5} or $2x10^{-5}$M 2,4-D.

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

Time (days)	2,4-D (M)	IAA oxidase activity* (µg)	Peroxidase activity** (OD)
0	-	-	-
5	10^{-5}	90	0.450
	$2x10^{-5}$	140	0.420
10	10^{-5}	210	0.520
	$2x10^{-5}$	325	0.580
15	10^{-5}	135	0.880
	$2x10^{-5}$	265	1.200
20	10^{-5}	175	0.720
	$2x10^{-5}$	300	1.100

*IAA oxidase activity: µg of IAA destroyed/mg dry weight of the tissue in 25 min.

**Peroxidase activity: It is expressed as the rate of increase in OD/min at 470 mµ.

Fig. 35 & 36. Changes in the activity of peroxidase (Fig. 35), and IAA oxidase (Fig. 36) enzymes released into the culture medium of Datura cell suspensions as influenced by 2,4-D concentrations.

Inoculum size: 300±30 mg tissue in 40 ml of modified MS medium supplemented with 10^{-5} or $2x10^{-5}$M 2,4-D.

Experimental details as given in Table 26.

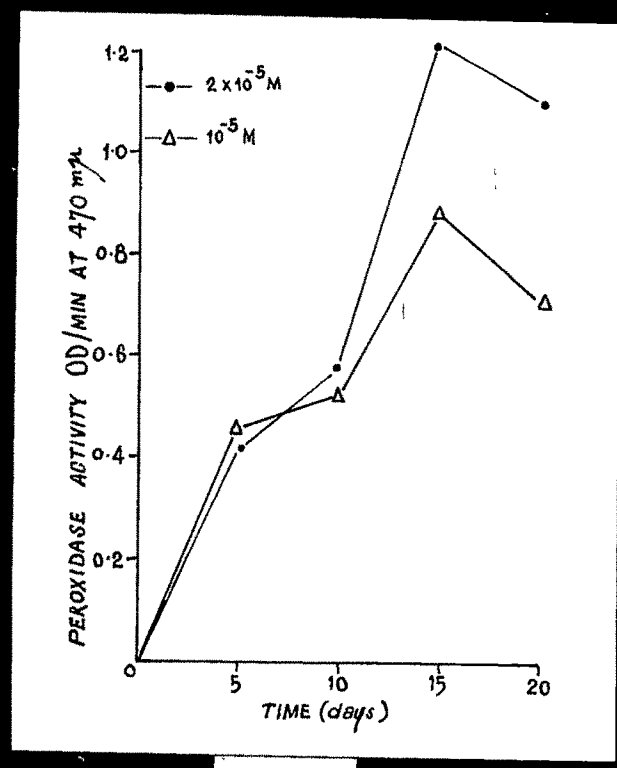
PEROXIDASE ACTIVITY OD/MIN AT 470 mμ
1·2
1·0
0·8
0·6
0·4
0·2
0
2 x 10⁻⁵ M
10⁻⁵ M
5
10
15
20
TIME (days)

Fig. 35

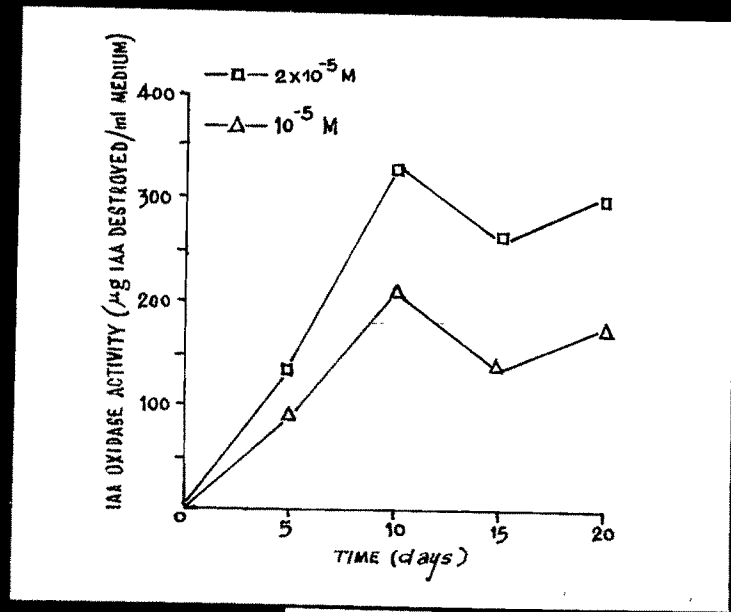
IAA OXIDASE ACTIVITY (μg IAA DESTROYED/ml MEDIUM)
400
300
200
100
0
2 x 10⁻⁵ M
10⁻⁵ M
5
10
15
20
TIME (days)

Fig. 36

Experiment 5-5 : Kinetin Effects on Peroxidase and Indoleacetic acid Oxidase Activities in Datura Cell Cultures

Modified Murashige and Skoog's medium (Table 3, Chpater II) was supplemented with 0, 10^{-6}, $2x10^{-6}$, 10^{-5} or $2x10^{-5}$M kinetin in addition to 2.0 mg/l 2,4-D and 2% sucrose. Measured aliquots of suspension (300±30 mg of tissue by fresh weight) were inoculated into flasks containing 40 ml medium each. The flasks were incubated under identical conditions as described earlier (Experiment 5-2). A fixed number of flasks was harvested at five days interval upto 20 days and the tissues of each treatment were pooled separately for the estimation of peroxidase and IAA oxidase activities as described in Materials and Methods, 7A and B (Chapter II).

The results presented in Table 27 and Fig. 37 clearly showed that there was considerable delay in the initiation of peroxidase activity at all kinetin treatments. Kinetin at higher concentrations was found to be inhibitory to peroxidase formation, whereas $2x10^{-6}$M kinetin concentration was stimulatory and the maximum

rate of peroxidase activity was recorded on day 15 at this concentration. The cells grown in absence of kinetin, though showed an initial sharp raise in activity, it declined after day 10 as was observed in case of cells grown in high kinetin (10^{-5} or $2x10^{-5}$M) media.

In presence of kinetin concentrations as presented in Table 28 and illustrated in Fig. 38 there was observed sharp raise in IAA oxidase activity during initial 5 days. The higher concentrations of kinetin (10^{-5} and $2x10^{-5}$M) were found to suppress the enzyme activity. The decrease in the rate of activity was also accompanied by the decrease in fresh weight yield of the tissue (Experiment 4-4). The maximum rate of activity was registered at $2x10^{-6}$M kinetin concentration which also supported maximum growth. Further at $2x10^{-6}$ and 10^{-6}M concentrations, though the activity declined on day 5 and 10 respectively, a further marked raise in the activity was observed after day 15.

The spurt in peroxidase activity at all kinetin levels during the pre-exponential growth period of 5 to 10 days corresponds to rapid polyphenol synthesis as recorded in previous experiments. There was on the other hand, vast difference in growth attained by the tissues at these kinetin levels as shown in Fig. 15, Experiment 4-4.

Table 27 : Effect of Kinetin Concentrations on Peroxidase Activity in Datura Cell Suspension Cultures

Inoculum : 300±30 mg tissue by fresh weight in 40 ml of modified MS medium (Table 3, Chapter II) supplemented with 0, 10^{-6}, $2x10^{-6}$, 10^{-5} or $2x10^{-5}$M kinetin.

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

Time (days)	Kinetin (M)	Peroxidase activity* (OD)
0	0	0.030
5	0	0.150
	10^{-6}	0.045
	$2x10^{-6}$	0.070
	10^{-5}	0.050
	$2x10^{-5}$	0.055
10	0	0.275
	10^{-6}	0.205
	$2x10^{-6}$	0.310
	10^{-5}	0.225
	$2x10^{-5}$	0.100
15	0	0.050
	10^{-6}	0.375
	$2x10^{-6}$	0.400
	10^{-5}	0.105
	$2x10^{-5}$	0.065
20	0	0.015
	10^{-6}	0.070
	$2x10^{-6}$	0.050
	10^{-5}	0.041
	$2x10^{-5}$	0.034

*Peroxidase activity: It is expressed as the rate of increase in OD/min at 470 mμ.

Table 28 : Effect of Kinetin Concentrations on IAA Oxidase Activity in *Datura* Cell Suspension Cultures

Inoculum : 300±30 mg tissue by fresh weight in 40 ml of modified MS medium (Table 3, Chapter II) supplemented with 0, 10^{-6}, $2x10^{-6}$, 10^{-5} or $2x10^{-5}$M Kinetin.

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

Time (days)	Kinetin (M)	IAA oxidase activity* (µg)	Specific activity**
0	0	85	1.250
5	0	135	5.326
	10^{-6}	230	10.410
	$2x10^{-6}$	175	12.020
	10^{-5}	150	8.526
	$2x10^{-5}$	110	6.340
10	0	100	5.002
	10^{-6}	160	16.426
	$2x10^{-6}$	300	22.426
	10^{-5}	145	12.246
	$2x10^{-5}$	86	3.420
15	0	50	0.826
	10^{-6}	200	26.680
	$2x10^{-6}$	180	18.482
	10^{-5}	140	12.650
	$2x10^{-5}$	65	2.120
20	0	45	2.200
	10^{-6}	275	28.600
	$2x10^{-6}$	225	18.620
	10^{-5}	130	16.000
	$2x10^{-5}$	75	3.890

*IAA oxidase activity: µg of IAA destroyed/mg dry weight of the tissue in 25 min.

**Specific activity: µg of IAA destroyed/µg protein.

Fig. 37 & 38. Changes in peroxidase (Fig. 37) and IAA oxidase (Fig. 38) activities of *Datura* cell suspensions grown at different kinetin concentrations.

Inoculum size: 300±30 mg tissue in 40 ml of modified MS medium supplemented with 0, 10^{-6}, $2x10^{-6}$, 10^{-5} or $2x10^{-5}$ M kinetin.

Experimental details as given in Table 27 and 28.

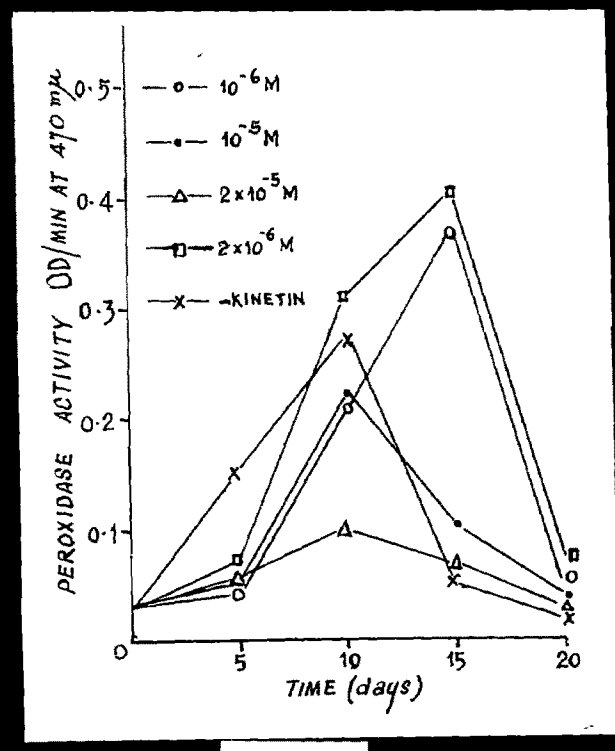
PEROXIDASE ACTIVITY OD/MIN AT 470 mμ
0·5
0·4
0·3
0·2
0·1
0
5
10
15
20
TIME (days)
— o — 10⁻⁶M
— • — 10⁻⁵M
— Δ — 2x10⁻⁵M
— □ — 2x10⁻⁶M
— x — -KINETIN

Fig. 37

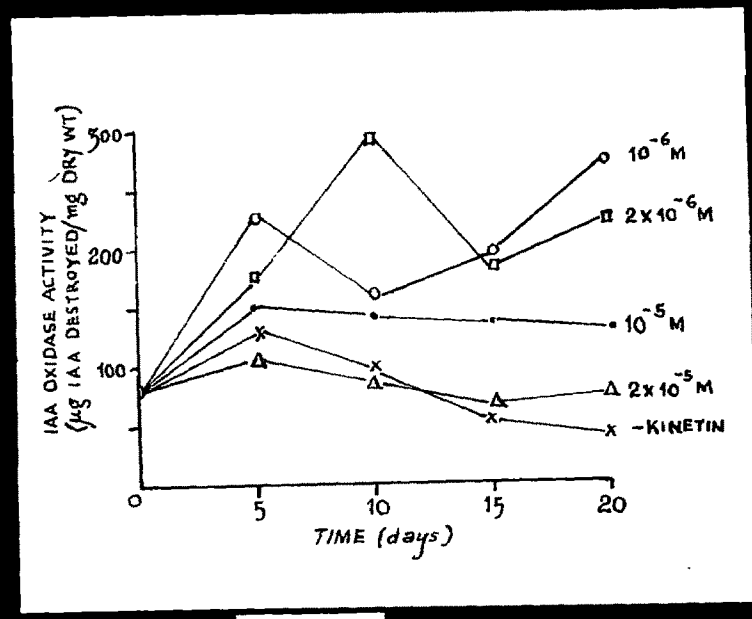
IAA OXIDASE ACTIVITY
(μg IAA DESTROYED/mg DRY WT)
300
200
100
0
5
10
15
20
TIME (days)
10⁻⁶M
2x10⁻⁶M
10⁻⁵M
2x10⁻⁵M
-KINETIN

Fig. 38

The kinetin effect on enhancing the IAA oxidase activity during the initial 5 days period (lag phase of growth) was similar to that of sucrose and 2,4-D. There was a fall in the IAA oxidase activity in the subsequent 5-10 day period at all kinetin levels except the optimal ($2x10^{-6}$M). This is unlike to that of sucrose and 2,4-D effects on IAA oxidase activity during this period of rapid polyphenol synthesis.

Experiment 5-6 : Release of Peroxidase and Indoleacetic Acid Oxidase Enzymes into the Culture Medium as influenced by Kinetin Concentrations

Modified MS medium (Table 3, Chapter II) was supplemented with 10^{-5} or $2x10^{-6}$M kinetin in addition to 2% sucrose and 2.0 mg/l 2,4-D. Measured aliquots of cell suspension (300±30 mg by fresh weight) was inoculated into each flask containing 40 ml of medium and the culture flasks were incubated in similar conditions as mentioned earlier (Experiment 5-2). A fixed number of replicate flasks was harvested at 5 days interval upto day 20 and the culture medium, separated from the cells, was centrifuged and dialyzed for assaying peroxidase and IAA oxidase activities. The enzyme activities were determined according to the standard

Table 29 : Effect of Kinetin on the Release of Peroxidase and IAA Oxidase Enzyme into the Culture Medium

Inoculum : 300±30 mg tissue by fresh weight in 40 ml of modified MS medium (Table 3, Chapter II) supplemented with $2x10^{-6}$ or 10^{-5} M kinetin.

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

Time (days)	Kinetin (M)	IAA oxidase activity* (µg)	Peroxidase activity** (OD)
0	-	-	-
5	$2x10^{-6}$	100	0.450
	10^{-5}	190	0.280
10	$2x10^{-6}$	185	0.520
	10^{-5}	165	0.306
15	$2x10^{-6}$	125	0.920
	10^{-5}	180	0.403
20	$2x10^{-6}$	180	0.740
	10^{-5}	215	0.422

*IAA oxidase activity: µg of IAA destroyed/mg dry weight of the tissue in 25 min.

**Peroxidase activity : It is expressed as the rate of increase in OD/min at 470 mµ.

Fig. 39 & 40. Changes in the activity of peroxidase (Fig. 39) and IAA oxidase (Fig. 40) enzymes released into the culture medium as influenced by kinetin concentrations.

Inoculum size: 300±30 mg tissue in 40 ml of modified MS medium supplemented with $2x10^{-6}$ or 10^{-5}M kinetin.

Experimental details as given in Table 29.

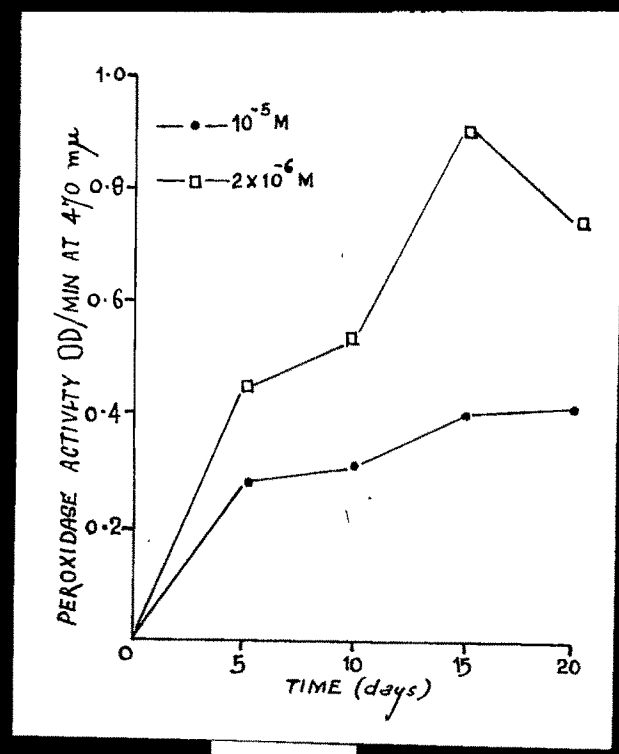
PEROXIDASE ACTIVITY OD/MIN AT 470 mμ
1·0
0·8
0·6
0·4
0·2
0
5
10
15
20
TIME (days)
10⁻⁵M
2x10⁻⁶M

Fig. 39

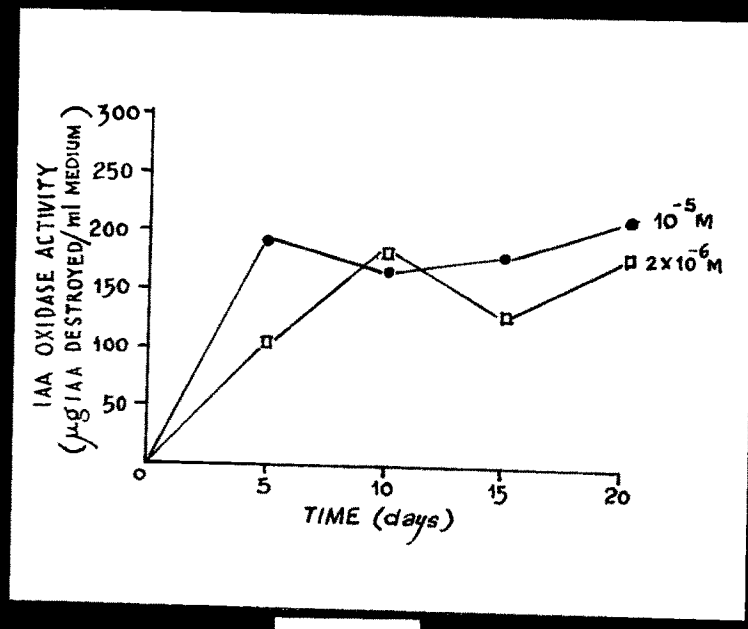
IAA OXIDASE ACTIVITY
(μg IAA DESTROYED/ml MEDIUM)
300
250
200
150
100
50
0
5
10
15
20
TIME (days)
10⁻⁵M
2x10⁻⁶M

Fig. 40

procedures given in Chapter II, Materials and Methods, 7A and B.

The results tabulated in Table 29 and illustrated in Fig. 39 and 40 clearly showed that kinetin and less appreciable influence on the leaching out of the enzymes as compared to sucrose and 2,4-D. At high concentration of kinetin (10^{-5}M) peroxidase secretion was much less than in the control (i.e. at $2x10^{-6}$M).

Large amount of IAA oxidase secretion was observed during lag phase at 10^{-5}M kinetin concentration and the decline in enzyme activity after day 10 was not very significant followed by a gradual raise (Fig. 40). In optimal kinetin medium, however, the IAA oxidase enhanced upto day 10 and another spurt was recognised after day 15.

Experiment 5-7 : Comparison of Growth and Polyphenol Content in Cassia and Cotton Tissues

Weighed amount (400±20 mg by fresh weight) of callus ~~into~~ pieces were inoculated into Erlenmeyer flasks containing 40 ml of standard MS medium (Table 3, Chapter II) supplemented with 2% sucrose, 2.0 mg/l 2,4-D and 0.4 mg/l kinetin. The culture flasks were incubated at constant temperature of 26±2°C in a continuously illuminated room. A fixed number

of replicate flaks was harvested at 3 days interval upto 15 days for determining fresh weights and for the estimation of total polyphenols as described in Materials and Methods, 5A, and 6A (Chapter II).

The results presented in Table 30 and illustrated in Fig. 41 and 42 clearly revealed that in the case of Cassia tissues there was no pronounced lag phase in growth. The growth was most rapid between 6 to 9 days and thereafter it slightly slowed down. However, in cotton tissues the marked lag phase for 3 days was followed by a sharp raise in growth which extended upto day 15, Furthermore, the final growth value (15 fold) in cotton tissues was considerably higher than that of Cassia tissues (13 fold).

When a comparison was drawn in the accumulation of polyphenol content between Cassia and cotton tissues, over 11 and 10 fold increase were recorded in respective tissues. This suggested that though there was no marked difference in polyphenol content measured in terms of fold increase, the total phenol content was considerably higher in Cassia tissues as compared to that in cotton. Initially Cassia tissues contained double the amount of polyphenols and it increased to over 3 times more than in cotton, per gram fresh weight, at the end of 15 days. In both the tissues

Table 30 : Comparison of Growth and Polyphenol Content in Cassia and Cotton Tissues*

Inoculum : 400±20 mg tissue by fresh weight in 40 ml of modified MS medium (Table 3, Chapter II) supplemented with 2% sucrose, 2 mg/l 2,4-D and 0.4 mg/l kinetin.

Incubation: 15 days in light at 26±2°C.

Time (days)	*Cassia* tissue			Cotton tissue		
	Fresh wt. (mg)	Polyphenols (µg/g Fr.wt.)	Polyphenols (µg/culture)	Fresh wt. (mg)	Polyphenols (µg/g Fr.wt.)	Polyphenols (µg/culture)
0	400±20	1170	468	400±20	534	200
3	708	1270	814	446	493	220
6	1512	1297	1380	1251	419	440
9	3480	990	2530	4022	377	980
15	5120	1098	5260	6256	323	2120

*Data represent average of six replicates.

Fig. 41. Comparison of growth in Cassia and Cotton callus cultures.

Inoculum size: 400±20 mg tissue in 40 ml of modified MS medium supplemented with 2% sucrose, 2 mg/l 2,4-D and 0.4 mg/l kinetin.

Experimental details as given in Table 30.

Fig. 42. Comparison of the changes in polyphenol content of Cassia and Cotton tissues.

Inoculum size: 400±20 mg tissue in 40 ml of modified MS medium supplemented with 2% sucrose, 2.0 mg/l 2,4-D and 0.4 mg/l kinetin.

Experimental details as given in Table 29.

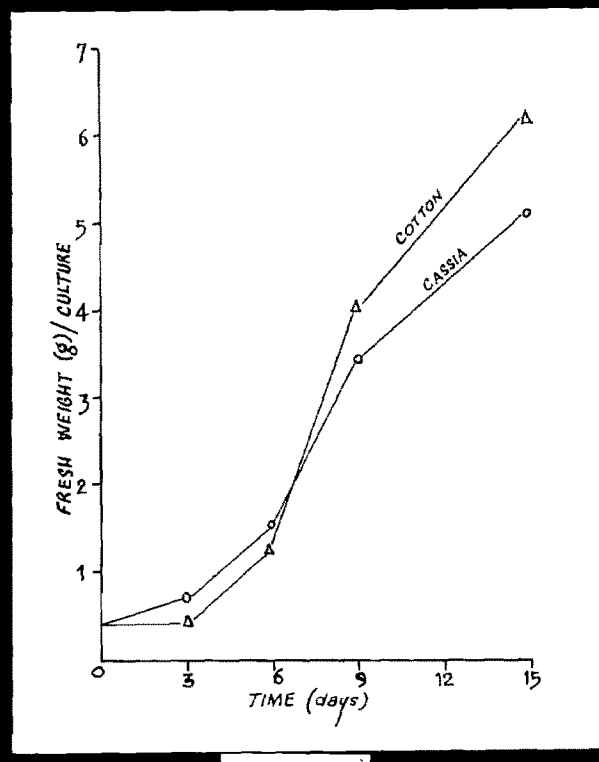
FRESH WEIGHT (g)/ CULTURE
7
6
5
4
3
2
1
0
3
6
9
12
15
TIME (days)
COTTON
CASSIA

Fig. 41

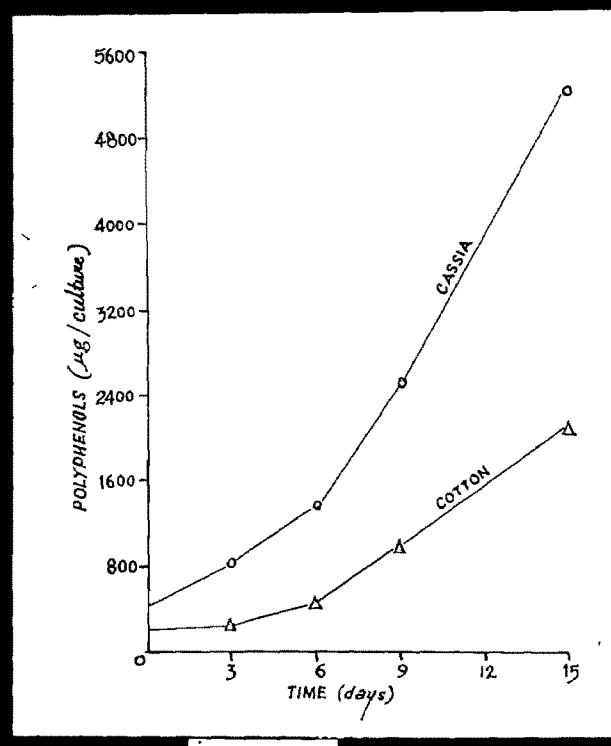
POLYPHENOLS (µg/culture)
5600
4800
4000
3200
2400
1600
800
0
3
6
9
12
15
TIME (days)
CASSIA
COTTON

Fig. 42

there was decrease in polyphenol content on unit fresh weight basis with passage of culture.

Experiment 5-8 : Effect of Sucrose Concentrations on Polyphenol Accumulation and the Development of Phenylalanine Ammonia-Lyase (PAL) Activity in Cassia Callus Cultures

Callus pieces weighing 400±20 mg by fresh weight were inoculated into Erlenmeyer flasks containing 40 ml of modified MS medium (Table 3, Chapter II) supplemented with 0.5, 2.0 or 6.0 per cent sucrose in addition to 0.4 mg/l kinetin and 2.0 mg/l 2,4-D.

The culture flasks were incubated for 15 days in light at a constant temperature of 26±2°C. A fixed number of replicate flasks was harvested at 3 days interval upto day 15 and the tissues of each treatment were pooled separately for the measurement of growth and total phenols and for assaying PAL activity as described in Chapter II, Materials and Methods, 5A, 6A and 7C.

The effect of sucrose concentration on growth, polyphenol production and the development of PAL activity are presented in Table 31 and illustrated in Fig. 43, 44 and 45.

Table 31 : Effect of Sucrose on Polyphenol Accumulation and Phenylalanine Ammonia-Lyase (PAL) Activity in Cassia Callus Cultures*

Inoculum : 400±20 mg tissue by fresh weight in 40 ml of modified MS medium (Table 3, Chapter II) supplemented with 0.5, 2.0 or 6.0% sucrose.

Incubation: 15 days in light of 26±2°C.

Time (days)	Sucrose concentrations (%)								
	0.5			2.0			6.0		
	Fresh wt. (mg)	Polyphenols (µg/culture)	PAL activity** (µm)	Fresh wt. (mg)	Polyphenols (µg/culture)	PAL activity** (µm)	Fresh wt. (mg)	Polyphenols (µg/culture)	PAL activity** (µm)
0	400±20	448	0.54	400±20	448	0.54	400±20	448	0.54
3	677	965	0.56	716	812	0.65	640	844	0.62
6	932	1746	0.62	1058	1375	0.92	973	1767	1.24
9	1172	1787	1.02	3560	2432	1.72	1566	2976	3.20
15	1670	1927	0.51	5240	5300	0.54	3523	7157	0.82

*Data represent average of six replicates.
**PAL activity: It is expressed as the micromoles of cinnamic acid produced at 40°C/hr/100 mg of acetone powder.

Fig. 43,44 & 45. Effect of sucrose levels on growth (Fig. 43), polyphenol content (Fig.44) and phenylalanine ammonia-lyase (PAL) activity (Fig. 45) in Cassia callus cultures.

Inoculum size: 400±20 mg tissue in 40 ml of modified MS medium supplemented with 0.5, 2.0 or 4.0% sucrose.

Experimental details as given in Table 3.

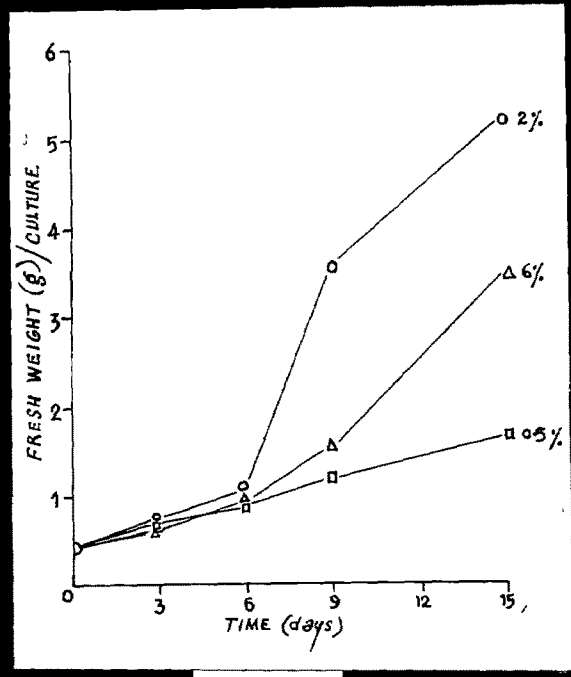

Fig. 43

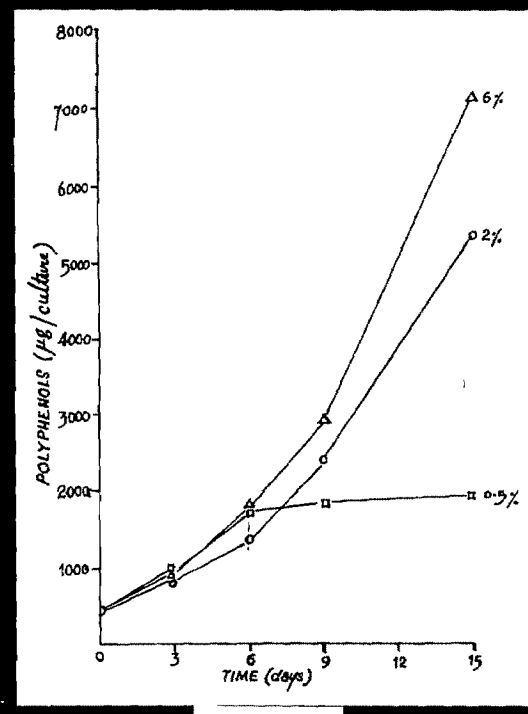

Fig. 44

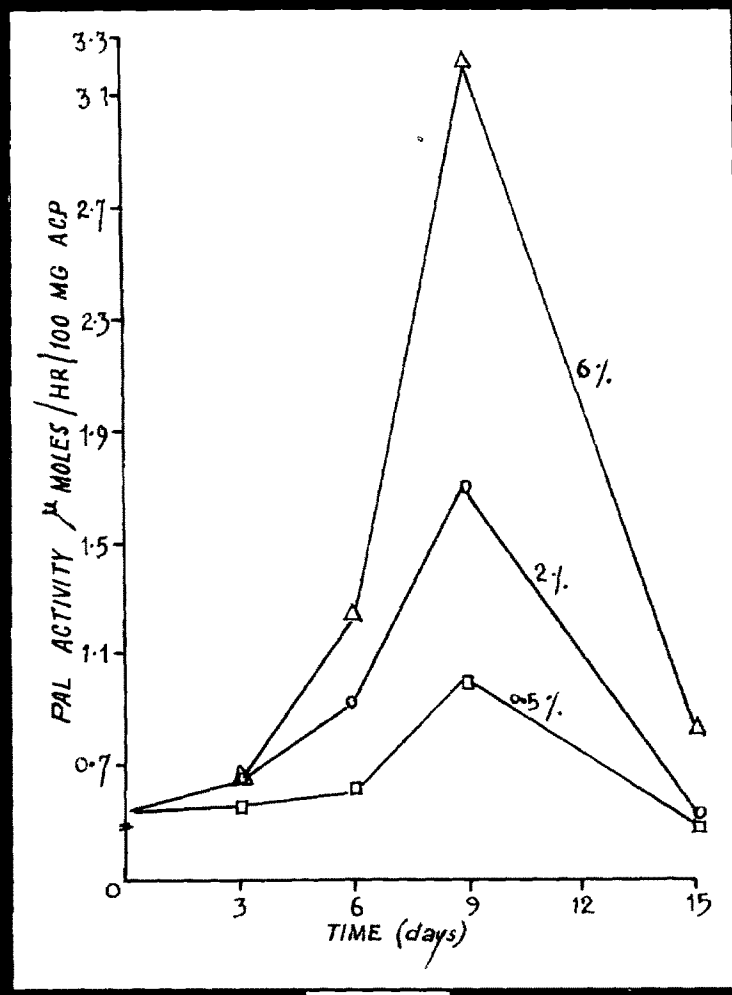

Fig. 45

Cassia cultures showed no pronounced lag phase in growth. The growth enhanced steadily upto day 6 in all treatments. At optimal sucrose level (2%) there was a sharp raise in growth during 6 to 9 days after which it slowed down.

The total polyphenols increased right from day '0'. In 2% and 6% sucrose media the maximum polyphenol production was recorded from 6 to 15 days. This period corresponded with exponential and post exponential growth phase for tissues grown on 2% sucrose medium. In the tissues incubated in 6% sucrose medium also this period (6-15 days) constituted the rapid growth phase. Very different picture was observed in case of low sucrose medium, where maximum polyphenol synthesis was registered during the early part of culture period (0 to 6 days) after which it remained stationary.

The PAL activity was low during the early stages of growth at all sucrose concentrations. However, the subsequent development corresponded quite closely with the rate of polyphenol synthesis upto day 9. Further, though the polyphenol production extended upto day 15, the PAL activity terminated on day 9. This suggested that a close correlation cannot be drawn between the production of polyphenols and

the development of PAL activity all through the culture period. Unlike growth, maximum polyphenol production and PAL activity were recorded in tissues grown on high sucrose (6%) medium.

Experiment 5-9 : 2,4-D Effects on Polyphenol Synthesis and the Development of Phenylalanine Ammonia-Lyase (PAL) Activity in Cassia Callus Cultures

Modified MS medium (Table 3, Chapter II) was supplemented with 0.2, 2.0 or 10 mg/l 2,4-D in addition to 0.4 mg/l kinetin and 2% sucrose. Callus pieces weighing 400±20 mg by fresh weight were inoculated into each Erlenmeyer flask containing 40 ml medium.

The culture flasks were incubated for 15 days at a constant temperature of 26±2°C in a continuously illuminated room. Fixed number of replicate flasks was harvested at 3 days interval upto 15 days and the tissues of each treatment were pooled separately for the determination of growth, total phenols and PAL activity as described in Chapter II, Materials and Methods, 5A, 6A and 7C.

The results are presented in Table 32 and illustrated in Fig. 46, 47 and 48. Clearly, at the highest concentration of 2,4-D (10 mg/l) growth, polyphenol synthesis and PAL activity were considerably reduced. Though the maximum growth was attained in 2.0 mg/l 2,4-D medium, the highest polyphenol synthesis as well as PAL activity were registered in tissues grown on 0.2 mg/l 2,4-D medium.

The PAL activity at high auxin concentrations was virtually absent upto day 3; then it suddenly raised to reach its peak on day 6 before gradually declining. The highest rates of PAL activities were recorded in 0.2 and 2.0 mg/ 2,4-D during 3 to 9 days period; while the highest polyphenol production was recorded ffom 6-15 days; thus, though the PAL activity terminated on days 6 or 9 depending upon auxin level in the medium, the polyphenol production continued to increase even after day 9. A rough correspondence existed between polyphenols accumulated and the PAL activity observed at 0.2 and 2.0 mg/l 2,4-D levels during the course of culture for 9 days.

Table 32 : Effect of 2,4-D on Polyphenol Synthesis and Phenylalanine Ammonia-Lyase (PAL) Activity in *Cassia* Callus Cultures*

Inoculum : 400±20 mg tissue by fresh weight in 40 ml of modified MS medium (Table 3, Chapter II) supplemented with 0.2, 2.0 or 10.0 mg/l 2,4-D.

Incubation: 15 days in light at 26±2°C.

Time (days)	2,4-D concentrations (mg/l)								
	0.2			2.0			10.0		
	Fresh wt. (mg)	Polyphenols (µg/culture)	PAL activity** (µm)	Fresh wt. (mg)	Polyphenols (µg/culture)	PAL activity** (µm)	Fresh wt. (mg)	Polyphenols (µg/culture)	PAL activity** (µm)
0	400±20	410	0.54	400±20	410	0.54	400±20	410	0.54
3	661	652	0.56	708	814	0.61	696	875	0.52
6	1063	1216	0.75	1512	1380	0.92	1290	1080	1.02
9	2545	4526	1.76	3480	2530	1.51	1812	1890	0.81
15	4789	8042	0.64	5120	5260	0.53	2782	3821	0.68

*Data represent the average of six replicates.
**PAL activity: It is expressed as the micromoles of cinnamic acid produced at 40°C/hr/100 mg of acetone powder.

Fig. 46, 47 & 48. 2,4-D effects on growth (Fig.46), polyphenol content (Fig.47) and phenylalanine ammonia-lyase (PAL) activity (Fig. 48) in Cassia callus cultures.

Inoculum size: 400±20 mg tissue in 40 ml of modified MS medium supplemented with 0.2,2.0 or 10.0 mg/l 2,4-D.

Experimental details as given in Table 32.

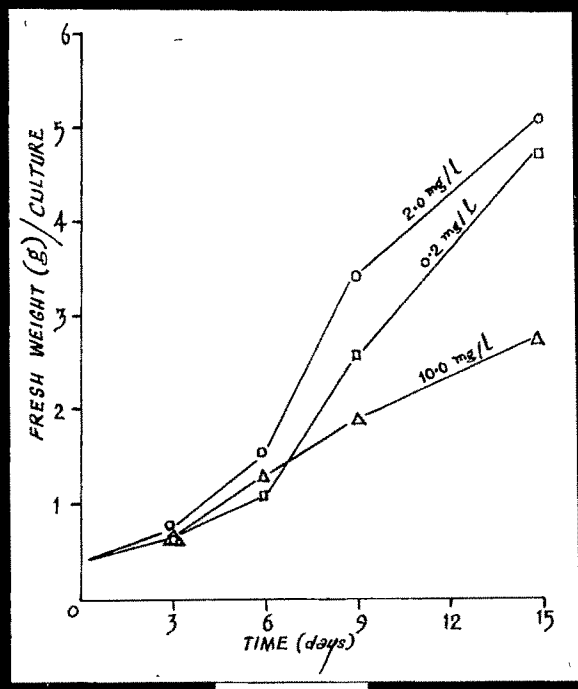

Fig. 46

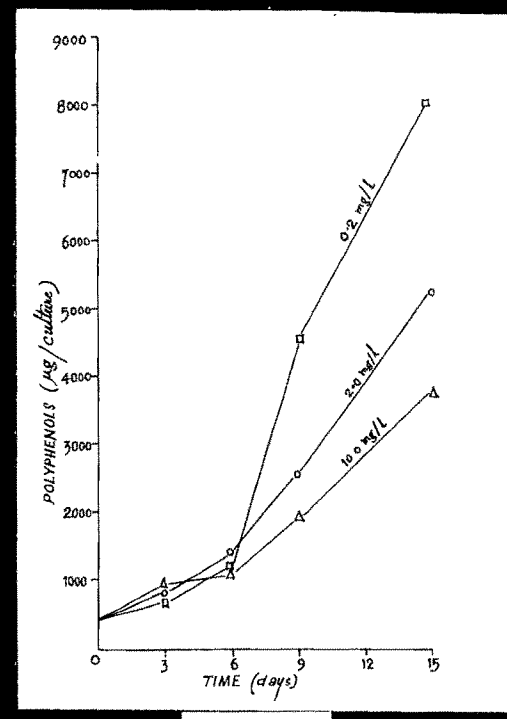

Fig. 47

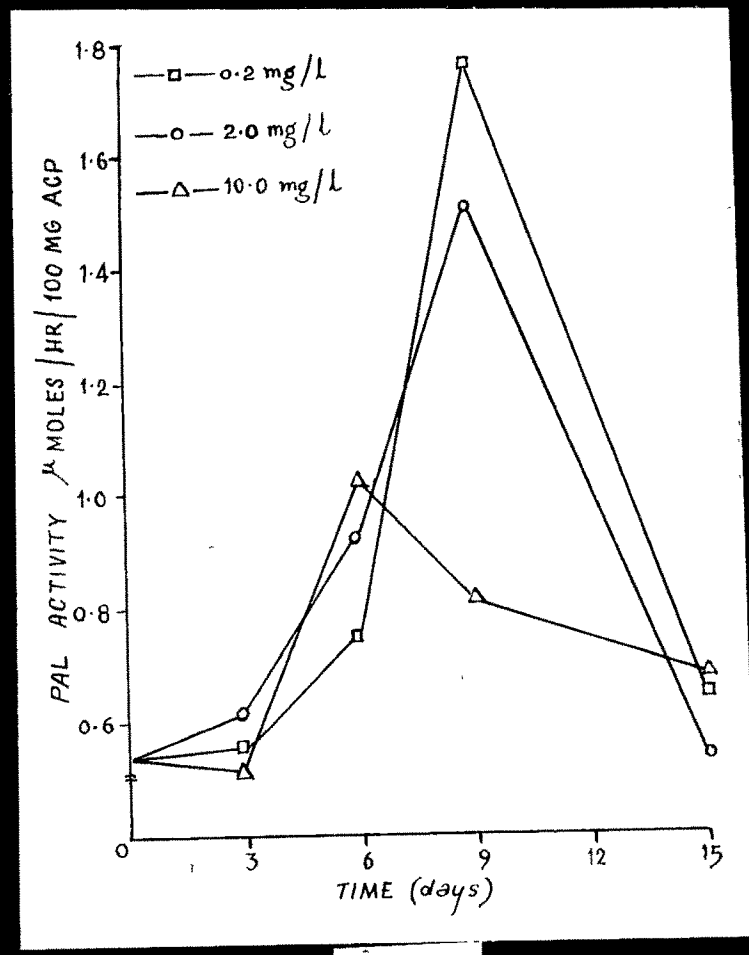

Fig. 48

DISCUSSION

When the effects of sucrose at different levels were tested on peroxidase and IAA oxidase activities (Experiment 5-1), it was observed that the peroxidase activity was high in sucrose medium from day 5 to day 15; whereas maximum IAA oxidase activity was recorded from 0 to 10 days. Clearly, the activities of peroxidase and IAA oxidase enzymes were high during the period of maximum polyphenol synthesis (5 to 10 days). In the most rapid phase of growth (10 to 15 days), while the peroxidase activity continued to increase, the IAA oxidase activity dropped. On the other hand, in the initial lag phase of growth though there was no pronounced rise in peroxidase activity, the IAA oxidase activity increased sharply. In both the cases the highest enzyme activity was recorded in high sucrose (4%) medium, however, their peak activities were attained on different days (IAA oxidase on day 10 and peroxidase on day 15).

The effects of 2,4-D on the pattern of IAA oxidase activity was not much different from that of sucrose. 2,4-D, however, markedly supressed the initiation of peroxidase activity during the lag phase of growth. Except

in auxin-free and low auxin (10^{-6}M) medium, there was sharp rise in peroxidase activity from day 5 to day 15, which, as noted earlier, corresponded with the period of rapid polyphenol synthesis (5 to 10 days) and also with the phase of rapid growth (10 to 15 days) of tissue. Barring the optimal (10^{-5}M) 2,4-D level which supported not only maximum growth and polyphenol production but also highest enzyme activities, the growth and IAA oxidase activity on one hand, and the polyphenol production and peroxidase activity on the other, varied very significantly with auxin concentrations in the medium. While the next best growth and IAA oxidase activities were attained in low (10^{-6}M) 2,4-D medium, the next highest polyphenol production and peroxidase activity were recorded in high ($2x10^{-5}$M) 2,4-D medium (Experiment 5-3).

Like 2,4-D, kinetin also had a delaying influence on the development of peroxidase activity. Higher kinetin ($2x10^{-5}$M) concentration tested supressed the enzyme activity, while the concentration optimal for growth ($2x10^{-6}$M) supported the maximum activity of peroxidase and IAA oxidase and also stimulated highest polyphenol production in the tissues (Experiment 5-5).

Higher kinetin levels (10^{-5} and $2x10^{-5}$M) also suppressed the IAA oxidase activity. IAA oxidase activity peak was attained in all treatments, except at optimal ($2x10^{-6}$M), on day 5. It was only in optimal kinetin concentration that the IAA oxidase increased during the rapid polyphenol synthesis (5 to 10 days). As observed in the case of sucrose and 2,4-D, there was another burst in IAA oxidase activity at optimal and sub-optimal kinetin concentrations on day 15. Further, the high kinetin levels (10^{-5} and $2x10^{-5}$M) which supressed the activities of peroxidase and IAA oxidase enzymes and also growth , had no corresponding effect on polyphenol production (Experiment 5-5).

The patterns of peroxidase enzyme released into the medium under the influence of sucrose (Experiment 5-2), 2,4-D (Experiment 5-4) and kinetin (Experiment 5-6) were very similar; the amount of enzyme released being considerably higher than that retained by the tissues. After appreciable release during the first 5 days, there was considerable retention of the enzyme by the tissues during 5 to 10 days - the period of maximum polyphenol production - in all three treatments. On the other hand, during the subsequent period of most rapid growth

(10 to 15 days) a sharp increase in the peroxidase activity was detected in the tissues as well as in the medium.

Contrary to peroxidase, the release of IAA oxidase into the medium was much less, particularly in the presence of 2,4-D and Kinetin. In general IAA oxidase released into the medium was less as compared to peroxidase. Kinetin, as compared to sucrose and 2,4-D, had least effect on IAA oxidase release, the enzyme being retained more in the tissue than its release into the medium. At optimal level (10^{-5}M) of 2,4-D greater amount of IAA oxidase was retained in the tissues than that released into the medium; however, at its higher level ($2x10^{-5}$M) about equal amount of enzyme activity was observed both in the tissues and in the medium. At optimal sucrose level more of IAA oxidase was retained in the tissues than released into the medium; whereas at supra-optimal level (4%) of sucrose large amount of enzyme was released into the medium.

Examination of the growth dynamics in Cassia and cotton tissues in experiment 5-7 revealed that cotton tissues showed marked lag phase, while in Cassia tissues growth enhanced from day 0. In both the cases, however,

rapid increase in fresh weight was recorded during 6 to 9 days period. At the end of incubation for 15 days, cotton registered slightly higher growth value than Cassia. The polyphenol production was observed all through the course of culture for 15 days in both the tissues, except that in case of cotton there was no polyphenol synthesis during the first 3 days.

When the relationship between growth, polyphenol synthesis and PAL activity was studied in Cassia tissues grown on different sucrose levels, it was found that while 2% sucrose supported maximum growth, it was in 6% sucrose that highest polyphenol synthesis and PAL activity were registered. Except in low (0.5%) sucrose medium, the polyphenol production occurred all through the course of culture for 15 days. The PAL activity, however, registered sharp increase from day 3 to day 9 before declining (Experiment 5-8).

The response of Cassia tissues to 2,4-D levels with respect to growth and total phenols was different from that of Datura. The maximum growth of Cassia tissues was supported by 2.0 mg/l 2,4-D in the medium, but highest total phenols and PAL activity were registered in tissues

incubated in 0.2 mg/l 2,4-D containing medium (Experiment 5-9). At all 2,4-D levels the total phenols continued to increase upto day 15, though the PAL activity terminated earlier (on day 6 or 9).