CHAPTER III

R E S U L T S

CHAPTER III : RESULTS

The experiments described in the present chapter were aimed to study the accumulation of phenolic compounds in Crotalaria juncea L. seedlings and their different parts during germination and in the callus tissues derived from the seedling. As indicated in the introduction (Chapter I), callus tissues derived from many plant parts could be grown in continuous culture on nutritive media which contain a utilizable sugar, inorganic salts and other growth promoting substances. Using Murashige and Skoog (1962) medium as absic composition, attempts were made to improve the growth of tissues by altering various cultural parameters and the same were tested for their ability to support polyphenol synthesis. The developmental patterns of few enzymes (i.e. peroxidase, IAA oxidase, PAL, TAL, p-coumarate : CoA ligase, phenylalanine transaminase and tyrosine transaminase) associated with the phenolic metabolism were also studied for the understanding of their possible role in the synthesis of phenolic materials. Further, PAL, a key enzyme in

the biosynthetic pathway leading to the phenyl-propanoid compounds, was purified from seedling and callus. *In vivo* and *in vitro* properties of PAL were examined and compared.

The experiments conducted to assess the influence of different cultural parameters on growth, accumulation of phenolic compounds and changes in the above mentioned enzymes are incorporated in the present chapter. Finally, the steps followed for the purification of PAL and the results obtained therein are also described.

The results obtained in above investigation are described under the following broad heads :-

A. Growth, Polyphenols and Related Enzymes in Germinating Seeds of *Crotalaria*.

B. Initiation and Growth of Seedling Callus of *Crotalaria*.

C. Nutritional Studies on Growth and Polyphenol Accumulation in *Crotalaria* Callus Cultures.

D. Progressive Changes in Growth, Accumulation of Phenolic Materials and the Development of

Related Enzyme Activities Under Different Cultural Conditions.

E. *In Vivo* and *In Vitro* L-Phenylalanine Ammonia-lyase Purification, Subunit Structure and Kinetic Properties.

A. CHANGES IN GROWTH, POLYPHENOLS AND THE DEVELOPMENT OF PEROXIDASE, IAA OXIDASE, PAL AND TAL ENZYMES DURING GERMINATION IN *CROTALARIA*

The seeds of *Crotalaria juncea* L. are very prompt in germination. After overnight soaking in water, all the parts of the seedling became visible within 24 hours. The seedlings are rich in phenolic compounds and also showed good activities of enzymes of phenylpropanoid biosynthesis (Shah *et al.*, 1976 a). Therefore, experiments were designed to study the changes in growth, phenolic materials and few enzymes (peroxidase, IAA oxidase, PAL and TAL) associated with the biosynthesis of these compounds during the course of germination for initial five days.

The seeds, surface sterilized in 0.1% (w/v) mercuric chloride solution, were kept on moistened

filter papers in petri dishes for germination in light at 28± 2°C as described earlier (Chapter II, Materials and Methods, 1). After every 24 hours, sets of 8 seedlings were harvested upto 120 hours. The seed-coats were removed and the different parts of seedling (i.e. cotyledon, hypocotyl and root) were separated carefully. Care was also taken to remove surface moisture using filter paper.

The progress of growth in seedling, cotyledon, hypocotyl and root is illustrated in Fig. A-1 (on fresh weight basis) and Fig. A-2 (on dry weight basis) and Table 3. There was rapid increase in growth in all the parts of seedling upto 72 hrs after which it slowed down. Overall 2.38 and 2.39 fold in seedling, 1.6 and 1.74 fold in cotyledon, 3.23 and 3.75 fold in hypocotyl and 3.0 and 4.5 fold increase in root were recorded in fresh and dry weights respectively during the course of incubation for 5 days. The rapid increase in growth from the beginning indicated prompt germination of the seeds. While maximum increase in fresh weight (on foldwise basis) was observed in hypocotyl, the maximum increase in dry weight (on foldwise basis) was recorded

in root. Clearly, the fresh weight increase of hypocotyl was due to its water-storing capacity.

The accumulation patterns of phenolic materials in seedling, cotyledon, hypocotyl and root are shown in Fig. A-3 and Table 3. In root polyphenols increased upto 96 hrs, whereas in other parts of seedling the increase was upto 72 hrs after which polyphenols synthesis declined. Though there was less accumulation of phenolic materials in the root and more in the cotyledon, foldwise increase was maximum in root, the overall increase being 2.0 fold as compared to 1.53 fold in cotyledon. Maximum production of phenolic compounds was registered during the period of most rapid growth (upto 72 hrs) in each of the parts of seedling.

When the data of phenolic production in each of the parts of seedling is plotted on unit basis (Fig. A-4 and Table 3), it became clear that synthesis of phenolic materials declined per mg dry weight in the parts in which overall increase in growth is maximum. Thus, in root and hypocotyl, where overall increase in dry weight being 4.5 and 3.75 folds respectively, continuous

Fig. A-1 and A-2. Changes in growth as measured by fresh (Fig. A-1) and dry (Fig. A-2) weights in seedling and its different parts during germination for initial 5 days.

- O - Seedling, -□- Cotyledon,
- Δ - Hypocotyl and - X - Root.

Experimental details as given in Table 3.

Fig. A-3 and A-4. Total (Fig. A-3) and relative amount (Fig. A-4) of polyphenol accumulation in seedling and each of its parts during initial 5 days of germination.

- O - Seedling, -□- Cotyledon,
- Δ - Hypocotyl and - X - Root.

Experimental details as given in Table 3.

Fig. A-5 and A-6. Progressive changes in total (Fig. A-5) and mean (Fig. A-6) peroxidase activity during germination for initial 5 days in seedling and its different parts.

- O - Seedling, - □ - Cotyledon,
- Δ - Hypocotyl and - X - Root.

Experimental details as given in Table 4.

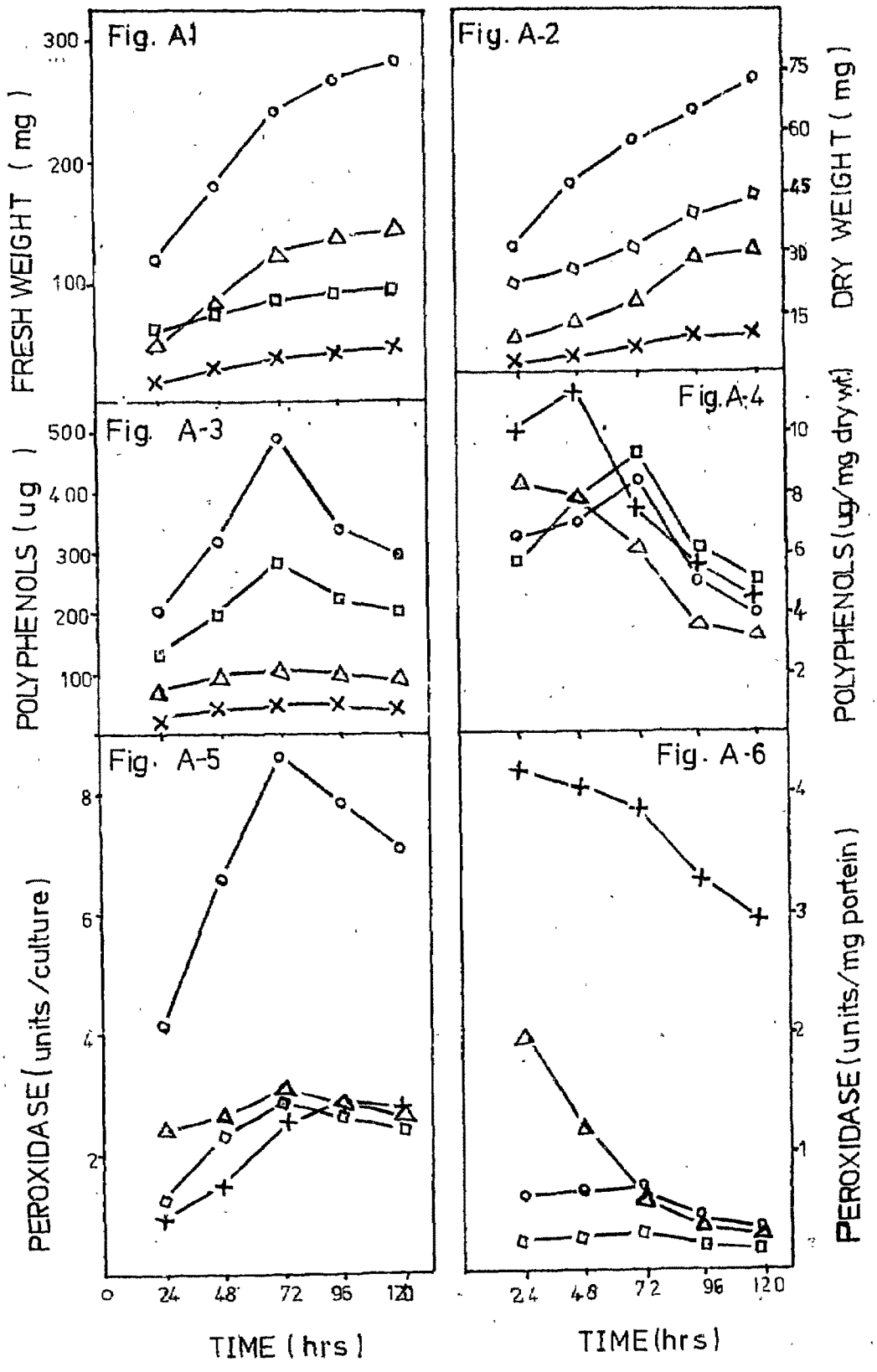


Fig. A1
Fig. A-2
Fig. A-3
Fig. A-4
Fig. A-5
Fig. A-6
FRESH WEIGHT (mg)
DRY WEIGHT (mg)
POLYPHENOLS (ug)
POLYPHENOLS (ug/mg dry wt)
PEROXIDASE (units /culture)
PEROXIDASE (units/mg portein)
TIME (hrs)
TIME (hrs)

Table 3 : Progressive Changes in Growth and Polyphenol Production in Crotalaria Seedling and its different Parts during Germination *

Incubation : 5 days in light at 28± 2°C.

Time (hrs.)	Seedlings				Cotyledons				Hypocotyls				Roots			
	Fresh Weight (mg)	Dry Weight (mg)	Polyphenols ug/ seedling	Polyphenols ug/mg dry wt.	Fresh Weight (mg)	Dry Weight (mg)	Polyphenols ug/ cot.	Polyphenols ug/mg dry wt.	Fresh Weight (mg)	Dry Weight (mg)	Polyphenols ug/ Hyp.	Polyphenols ug/mg dry wt.	Fresh Weight (mg)	Dry Weight (mg)	Polyphenols ug/ Root	Polyphenols ug/mg dry wt.
24	119 (± 1.1)	31 (± 0.4)	202	6.5	60 (± 1.0)	23 (± 0.3)	135	5.8	44 (± 0.6)	8 (± 0.15)	67	8.3	15 (± 0.2)	2 (±0.05)	20	10.0
48	178 (± 1.8)	46 (± 0.6)	324	7.0	71 (± 0.9)	26 (± 0.6)	200	7.7	81 (± 1.1)	12 (± 0.09)	94	7.8	26 (± 0.4)	3 (±0.03)	34	11.3
72	242 (± 1.4)	58 (± 0.3)	498	8.3	85 (± 1.2)	31 (± 0.4)	285	9.2	121 (± 2.0)	17 (± 0.2)	104	6.1	36 (± 0.7)	5 (±0.09)	37	7.4
96	267 (± 2.0)	65 (± 0.8)	341	5.0	91 (± 1.3)	39 (± 0.6)	225	6.1	135 (± 1.8)	28 (± 0.4)	100	3.6	41 (± 0.9)	8 (±0.08)	44	5.5
120	283 (± 1.9)	74 (± 0.9)	303	4.0	96 (± 2.1)	44 (± 0.9)	206	5.1	142 (± 1.2)	30 (± 0.6)	96	3.2	45 (± 1.0)	9 (±0.1)	40	4.4

* Data represent average of six replicates.

Figures in the parenthesis represent standard error.

decrease in phenolic compounds was recorded from the beginning in hypocotyl and after 48 hrs in root. On the other hand, seedling and cotyledon, where overall increase in dry weights were only 2.39 and 1.75 folds respectively, showed a pattern similar to that of phenolic production per individual part. In these parts, initially there was rapid increase in polyphenol synthesis upto 72 hrs after which it declined, the fall being not as pronounced as in the case of hypocotyl and root.

The developmental pattern of peroxidase activity in seedling and its different parts is presented in Fig. A-5 and Table 4. Though maximum production of phenolic compounds was recorded in cotyledon, highest development of peroxidase activity was registered in hypocotyl. However, the patterns of enzyme activity in individual parts of seedling showed close correlation with the patterns of polyphenol accumulation. Further, root showed the maximum overall increase in peroxidase activity and also in polyphenol production, whereas it was minimum in hypocotyl. The maximum enzyme activity

was recorded during the period of most rapid growth in each of the parts of seedling; the peak in the enzyme activity being observed on day 3 in seedling, cotyledon and hypocotyl and on day 4 in root.

Evaluation of the data on relative development of peroxidase activity is illustrated in Fig. A-6 and Table 4. The development of the enzyme activity in seedling, cotyledon and hypocotyl revealed similar patterns as those of polyphenol production on unit basis. In seedling and cotyledon enzyme activity increased upto day 3 after which it declined. On the other hand, in hypocotyl the activity showed continuous decrease from the beginning. In root, though the accumulation of phenolic materials increased initially upto day 2 and then declined, the peroxidase activity declined from the beginning. However, the developmental pattern of peroxidase and the accumulation of phenolic compounds in root showed close correlation after day 2.

The progressive changes in the development of IAA oxidase activity in seedling and its different parts is shown in Fig. A-7 and Table 4. Unlike peroxidase

Table 4 : Progressive Changes in Peroxidase and IAA oxidase Activities during Germination in Crotalaria Seeds and its different parts

Incubation : 5 days in light at 28 ± 2°C.

Time (hrs.)	Seedlings				Cotyledons				Hypocotyls				Roots			
	Peroxidase		IAA oxidase		Peroxidase		IAA oxidase		Peroxidase		IAA oxidase		Peroxidase		IAA oxidase	
	Units/ seedling	units/mg protein	Units/ seedlings	units/mg protein	units/ cot.	units/mg protein	units/ cot.	units/mg protein	units/ Hyp.	units/mg protein	units/ Hyp.	units/mg protein	units/ Root	units/mg protein	units/ root	units/mg protein
24	4.18	0.62	7.41	1.09	1.22	0.23	1.89	0.36	2.40	1.98	1.23	1.02	0.92	4.18	4.42	20.09
48	6.64	0.64	11.83	1.14	2.30	0.26	3.40	0.39	2.65	1.19	2.92	1.31	1.48	4.04	5.51	15.05
72	8.66	0.66	17.90	1.37	2.84	0.28	4.40	0.43	3.10	0.60	7.30	1.42	2.48	3.87	6.90	10.78
96	7.92	0.45	23.41	1.30	2.60	0.21	4.20	0.33	2.86	0.38	11.51	1.52	2.86	3.28	7.81	8.96
120	7.13	0.36	24.05	1.21	2.46	0.18	4.00	0.29	2.62	0.33	13.07	1.63	2.75	2.94	7.20	7.69

activity in seedling and hypocotyl where the peak was attained on day 3, there was continuous increase in IAA oxidase activity in these two parts. However, in cotyledon and root the pattern of development of IAA oxidase activity was very much similar to that of peroxidase; the peak being observed on day 3 in cotyledon and on day 4 in root for both the enzymes.

Fig. A-8 and Table 4 illustrates IAA oxidase activity on unit basis. In root there was continuous decrease in IAA oxidase activity per mg protein whereas in hypocotyl there was continuous increase from the beginning. The seedling and cotyledon showed a peak on day 3 and the pattern of the development of IAA oxidase corresponded with that of peroxidase and polyphenols. The pattern of root IAA oxidase was also very much similar to that of root peroxidase. However, the hypocotyl pattern was entirely different for both the enzymes.

The progressive changes in PAL activity in seedling and its different parts is presented in Fig. A-9 and Table 5. PAL activity in all the parts increased rapidly to reach a peak on day 3 in seedling,

cotyledon and hypocotyl and on day 4 in root. Thereafter the activity declined rapidly in all the cases except the root. Individual development of PAL in each of the parts of seedling showed close correlation with the synthesis of phenolic compounds.

The data are replotted in Fig. A-10 (Table 5) to illustrate the mean PAL content of the seedling and its parts. The root and hypocotyl showed decrease in the enzyme from the beginning, whereas PAL enhanced upto day 2 in cotyledon and upto day 3 in seedling. When the development of PAL activity per mg of protein was compared with the mean rate of accumulation of phenolic materials, it became apparent that maximum rate of phenolic synthesis generally occurred after the development of maximum enzyme activity, except in the case of whole seedling where the two peaks synchronized.

The development of TAL activity in seedling and its parts is shown in Fig. A-11 and Table 5. The TAL activity reached a peak 24 hours earlier than PAL activity in all the cases. After initial rapid increase, TAL activity terminated on day 2 in seedling, hypocotyl

Fig. A-7 and A-8. Progressive changes in specific (Fig. A-8) and total (Fig. A-7) activity of IAA oxidase in seedling and its different parts during initial 5 days of germination.

- O - Seedling, -□ - Cotyledon,
- △ - Hypocotyl and - X - Root.

Experimental details as given in Table 4.

Fig. A-9 to A-12. The development of PAL (Fig. A-9) and TAL (Fig. A-11) and the changes in specific activity of PAL (Fig. A-10) and TAL (Fig. A-12) enzymes during germination for initial 5 days.

- O - Seedling, -□ - Cotyledon,
- △ - Hypocotyl and -X- Root.

Experimental details as given in Table 5.

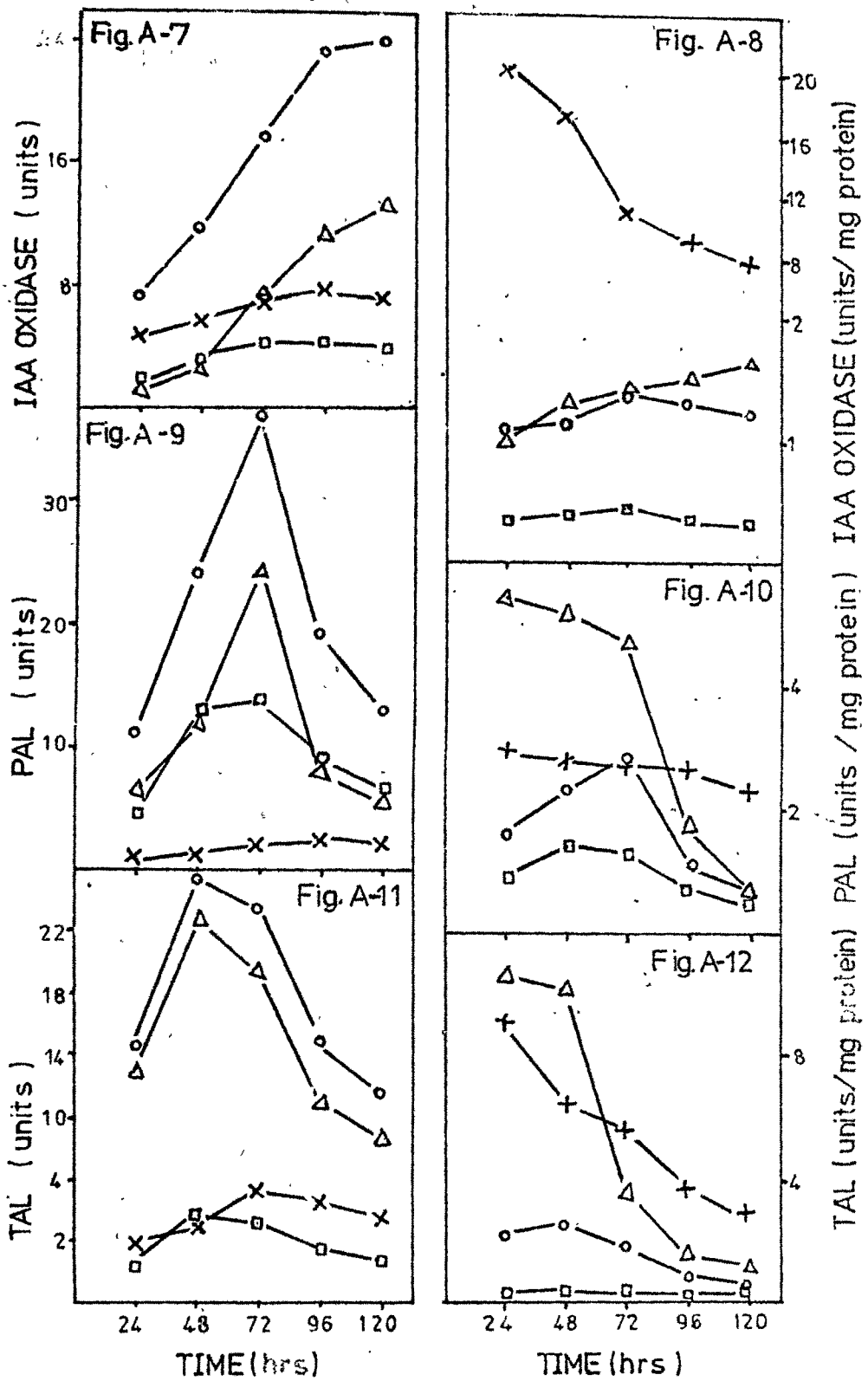


Fig. A-7
IAA OXIDASE (units)
16
8
Fig. A-8
20
16
12
8
2
1
IAA OXIDASE (units/ mg protein)
Fig. A-9
PAL (units)
30
20
10
Fig. A-10
4
2
PAL (units / mg protein)
Fig. A-11
TAL (units)
22
18
14
10
4
2
Fig. A-12
8
4
TAL (units/mg protein)
24
48
72
96
120
TIME (hrs)
24
48
72
96
120
TIME (hrs)

Table 5 : Progressive Changes in the Development of Phenylalanine Ammonia-Lyase (PAL) and Tyrosine Ammonia-Lyase (TAL) Activities in Crotalaria Seedling and its Different Parts during Germination.

Incubation : 5 days in light at 28± 2°C.

Time	Seedlings				Cotyledons				Hypocotyls				Roots			
	PAL		TAL		PAL		TAL		PAL		TAL		PAL		TAL	
(hrs.)	units/ seedling	units/mg protein	units/ seedlings	units/mg protein	Units/ cot.	units/mg protein	units/ cot.	units/mg protein	units/ Hyp.	units/mg protein	units/ Hyp.	units/mg protein	units/ Root	units/mg protein	units/ Root	units/mg protein
24	11.15	1.64	14.8	2.18	4.80	0.91	1.20	0.23	6.60	5.45	12.80	10.57	0.65	2.95	1.98	9.00
48	20.92	2.31	25.86	2.50	12.78	1.46	2.84	0.32	11.58	5.20	22.68	10.18	1.06	2.85	2.34	6.39
72	37.68	2.89	23.51	1.80	13.60	1.34	2.55	0.25	24.20	4.70	19.36	3.49	1 78	2.76	3.60	5.62
96	19.30	1.07	14.90	0.82	9.10	0.72	1.82	0.14	8.10	1.07	10.80	1.42	2.26	2.60	3.28	3.76
120	12.95	0.65	11.66	0.59	6.72	0.48	1.44	0.10	5.68	0.71	8.52	1.06	2.17	2.32	2.70	2.88

and cotyledon and on day 3 in root. As compared to the PAL activity, TAL activity was low in seedling, hypocotyl and cotyledon but high in the root.

Like PAL, when the specific activity of TAL is plotted (Fig. A-12 and Table 5), it became clear that maximum rates of phenolic synthesis reached after the development of maximum enzyme activity. For example, in seedling the maximum enzyme activity (2.50 μmoles p-coumarate/mg protein/hr) occurred between days 1-2, while maximum rates of phenolic synthesis (8.3 μg of chlorogenic acid/mg dry wt.) was reached 24 hours later.

SUMMARY

It is clear from the above experiments that seeds of Crotalaria juncea L. are very prompt in germination. The seedlings are rich in phenolic compounds and showed high activity of enzymes related to phenolic materials. The maximum synthesis of polyphenols and the development of different enzymes was observed during the period of most rapid growth in each of the parts of seedling. The developmental patterns of different enzymes and the accumulation of phenolic compounds indicated the signifi-

cant role of these enzymes in the synthesis of polyphenols. The possible mechanism of action of these enzymes in the biosynthesis of phenolic materials is discussed later (Chapter IV).

B. INITIATION AND GROWTH OF SEEDLING CALLUS OF CROTALARIA.

1. INITIATION OF CALLUS AND SUSPENSION CULTURES

In order to determine the most suitable medium to initiate callus from the Crotalaria seedling and for its subsequent indefinite growth, 4 days old aseptically grown seedlings were cut into pieces and the segments were placed on various solid media in Erlenmeyer flasks. The media were prepared as described in Chapter II, Materials and Methods 2 A. Murashige and Skoog (1962) basal medium (Table 1) with the following supplements was tested for callus induction :-

a) 2,4-D (2.0 mg/l and 4.0 mg/l),

b) IAA (2.0 mg/l and 4.0 mg/l),

c) NAA (2.0 mg/l and 4.0 mg/l),

d) 2,4-D (2.0 mg/l or 4.0 mg/l) and kinetin (2.0 mg/l),

e) IAA (2.0 mg/l or 4.0 mg/l) and kinetin (2.0 mg/l),

f) NAA (2.0 mg/l or 4.0 mg/l) and kinetin (2.0 mg/l),

g) 2,4-D (2.0 mg/l or 4.0 mg/l), IAA (2.0 mg/l or 4.0 mg/l) and kinetin (2.0 mg/l or 4.0 mg/l), and

h) 2,4-D (2.0 mg/l or 4.0 mg/l), NAA (2.0 mg/l or

4.0 mg/l) and kinetin (2.0 mg/l or 4.0 mg/l).

The flasks were incubated in continuous light at 26± 2°C. Callus was formed from the seedling on all the media tested. However, the initiation was best on the medium containing 2.0 mg/l 2,4-D, 2.0 mg/l IAA and 2.0 mg/l kinetin (Fig. B-1). Within four weeks of incubation, the seedling segments were completely over-grown by a mass of callus tissue (Fig. B-2).

Cell suspensions were also obtained from the callus tissues as described in Chapter II, Materials and Methods, 4 B. The suspension cultures so initiated were maintained by subculturing every 4 weeks. 5 ml aliquots of cell suspension were pipetted out into 40 ml of freshly made liquid medium of the same composition.

2. EFFECT OF HORMONES ON CALLUS GROWTH

Experiments were then conducted to find out the specific hormonal requirement for most rapid and continuous growth. The callus, after four passages on the medium on which it was initiated (MS medium on

Fig. B-1. Initiation of callus from Crotalaria seedling on MS medium supplemented with 2.0 mg/l 2,4-D, 2.0 mg/l IAA and 2.0 mg/l kinetin.

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

Other experimental details as given in the text.

Fig. B-2. Growth of Crotalaria callus after 4 weeks on MS medium supplemented with 2.0 mg/l 2,4-D, 2.0 mg/l IAA nad 2.0 mg/l kinetin.

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

Other experimental details as given in the text.

Fig. B -1.

Fig. B -2.

supplemented with 2.0 mg/l 2,4-D, 2.0 mg/l IAA and 2.0 mg/l kinetin), was transferred to MS medium with several hormonal combinations for the determination of most suitable synthetic medium for the growth of C. juncea seedling callus.

Tissue masses each weighing 300±30 mg (fresh weight) were inoculated in 150 ml Erlenmeyer flasks containing 30 ml of MS medium (Chapter II, Table 1) supplemented with :-

a) 2,4-D (2.0 mg/l),

b) NAA (2.0 mg/l),

c) IAA (2.0 mg/l),

d) 2,4-D (2.0 mg/l) and kinetin (0.4 mg/l)

e) NAA (2.0 mg/l) and kinetin (0.4 mg/l), and

f) IAA (2.0 mg/l) and kinetin (0.4 mg/l).

The growth as measured by increase in fresh and dry weights after incubation of the cultures for a period of four weeks in light at 26± 2°C is presented in Fig. B-3 and Table 6. The growth of the callus was found to be most rapid on the medium containing

Table 6 : Effect of Different Hormones on Growth of Callus Cultures *

Inoculum : 300± 30 mg tissue by fresh weight on 30 ml MS medium supplemented with

(A) 2.0 mg/l 2,4-D,

(B) 2.0 mg/l NAA,

(C) 2.0 mg/l IAA,

(D) 2.0 mg/l 2,4-D and 0.4 mg/l kinetin,

(E) 2.0 mg/l NAA and 0.4 mg/l kinetin, and

(F) 2.0 mg/l IAA and 0.4 mg/l kinetin.

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

Medium	Fresh Weight (mg/culture)	Dry Weight (mg/culture)
A	6770 (± 58)	229 (± 7.8)
B	5115 (± 37)	211 (± 1.8)
C	3918 (± 32)	182 (± 1.1)
D	5611 (± 25)	205 (± 3.0)
E	4810 (± 36)	201 (± 3.1)
F	3722 (± 23)	176 (± 2.4)

* Data represents average of six replicates.
Figures in the paranthesis represent standard errors.

Table 7 : Effect of Different Levels of 2,4-D on Growth of Callus Cultures*

Inoculum : 300± 30 mg tissue by fresh weight on MS medium supplemented with 0.0, 0.2, 1.0, 2.0, 5.0 and 10.0 mg/l 2,4-D.

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

2,4-D conc. (mg/l)	Fresh Weight (mg/culture)	Dry Weight (mg/culture)
0.0	927 (± 31)	53 (± 1.5)
0.2	4625 (± 43)	156 (± 3.3)
1.0	5235 (± 52)	169 (± 4.1)
2.0	6770 (± 58)	228 (± 7.8)
5.0	4180 (± 48)	133 (± 3.1)
10.0	2689 (± 50)	86 (± 2.3)

* Data represent average of six replicates.

Figures in the paranthesis represent standard errors.

Fig. B-3. Effect of different hormones on growth of the callus cultures.

Inoculum : 300± 30 mg tissue by fresh weight on MS medium supplemented with :

A. 2.0 mg/l 2,4-D,

B. 2.0 mg/l NAA,

C. 2.0 mg/l IAA,

D. 2.0 mg/l 2,4-D and 0.4 mg/l kinetin,

E. 2.0 mg/l NAA and 0.4 mg/l kinetin, and

F. 2.0 mg/l IAA and 0.4 mg/l kinetin.

Experimental details as given in Table 6.

Fig. B-4. Influence of various concentrations of 2,4-D on growth of the seedling callus.

Inoculum : 300± 30 mg tissue by fresh weight on MS medium supplemented with 0.0, 0.2, 1.0, 2.0, 5.0 or 10.0 mg/l 2,4-D.

Experimental details as given in Table 7.

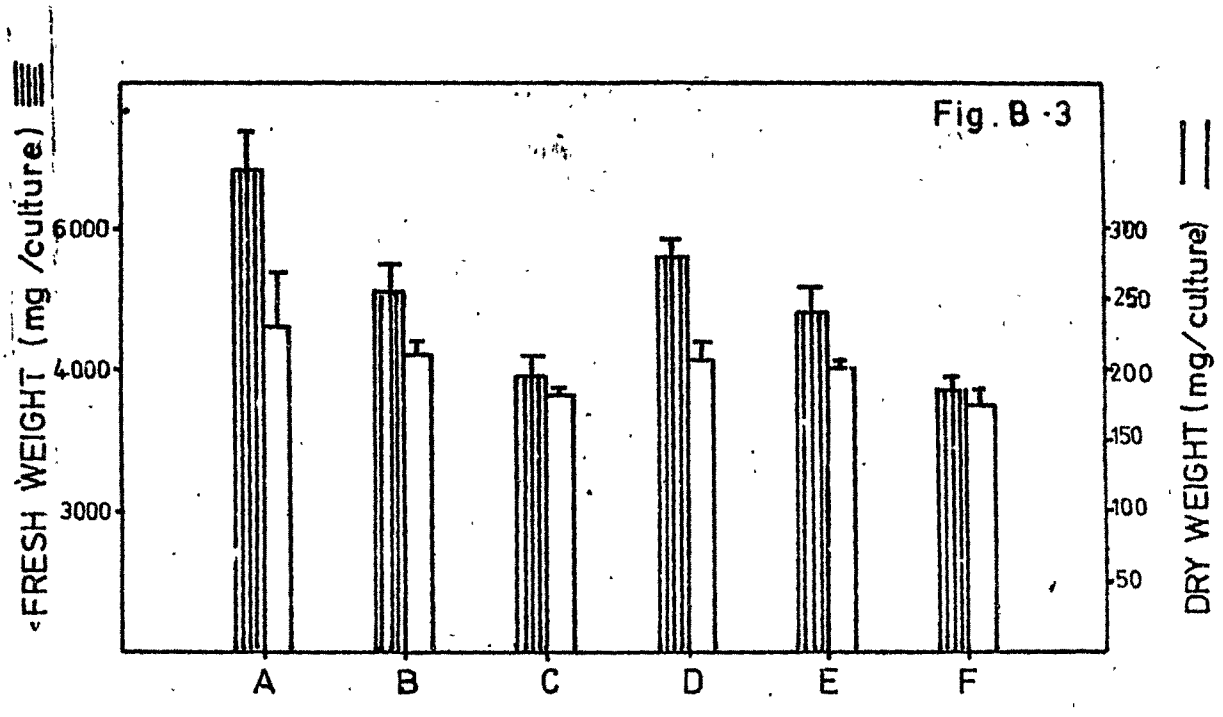


Fig. B-3
FRESH WEIGHT (mg /culture)
DRY WEIGHT (mg/culture)
6000
4000
3000
300
250
200
150
100
50
A
B
C
D
E
F

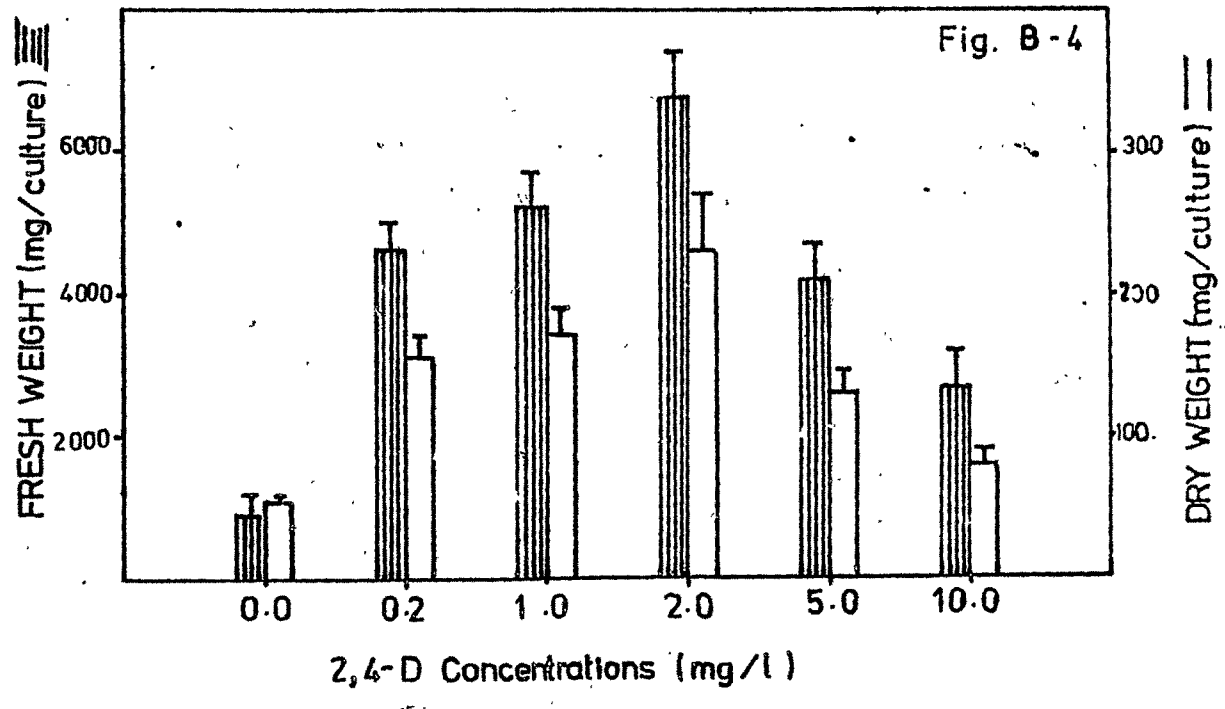


Fig. B-4
FRESH WEIGHT (mg/culture)
DRY WEIGHT (mg/culture)
6000
4000
2000
300
200
100
0.0
0.2
1.0
2.0
5.0
10.0
2,4-D Concentrations (mg/l)

2.0 mg/l 2,4-D (22.5 fold increase in fresh weight), as compared with the growth of the callus on other media (17 fold on NAA and 13 fold on IAA containing medium). When kinetin was incorporated into the medium along with 2,4-D, there was reduction in the growth of the callus (18.7 fold increase in fresh weight as compared to 22.5 fold on 2,4-D medium). Kinetin failed to enhance callus growth in presence of other auxins (NAA, IAA) also. Clearly, Crotalaria tissues have no exogenous requirement for cytokinin as they grew quite satisfactorily on the defined medium supplemented with 2.0% sucrose and 2.0 mg/l 2,4-D alone.

Further experiments were conducted to study the optimal requirement of 2,4-D concentration for best callus growth. The auxin concentrations tested were : 0.0, 0.2, 1.0, 2.0, 5.0 and 10.0 mg/l. The results obtained after incubation for four weeks in light at $26 \pm 2^{\circ}C$ are illustrated in Fig. B-4 and Table 7. As can be seen, 2.0 mg/l 2,4-D supported maximum growth as measured by two different growth parameters. Growth increased with the increase in the 2,4-D concentration upto 2.0 mg/l and with further increase in the auxin concentration growth declined.

3. GROWTH CURVE OF *CROTALARIA* CALLUS AND SUSPENSION CULTURES

After several passages on Murashige and Skoog (1962) medium (Table 1, Chapter II), weighed (300± 30 mg fresh weight) masses of callus were transferred to Erlenmeyer flasks containing 30 ml of freshly made culture medium. The cultures were incubated in light at 26± 2°C. For the growth curve of suspension culture, measured aliquots of *Crotalaria* cell suspensions weighing approximately 300± 30 mg by fresh weight were transferred to 40 ml of the freshly made liquid culture medium into 150 ml Erlenmeyer flasks. The flasks were incubated on a horizontal rotary shaker in constant light at 26± 2°C.

Six replicates were harvested at intervals of 2 days for initial 10 days and at intervals of 5 days from 10 to 30 days to determine the growth of callus and suspension cultures. The results are presented in Fig. B-5 (fresh weights) and Fig. B-6 (dry weights) and Table 8 and Table 9. The growth, measured in terms of fresh and dry weights and plotted against time, showed the usual type of sigmoid curve for both the callus and

Table 8 : Standard Growth Curve of *Crotalaria* Callus Culture Grown on Synthetic Medium*

Inoculum : 300± 30 mg tissue by fresh weight (dry weight 12.0 mg) on 30 ml of modified Murashige and Skoog medium (Table 1, Chapter II) supplemented with 2.0% sucrose and 2.0 mg/l 2,4-D.

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

Time (days)	Fresh Weight (mg/culture)	Dry Weight (mg/culture)
0	300 (± 30)	11.00 (± 2.8)
2	308 (± 35)	12.51 (± 3.1)
4	366 (± 33)	14.00 (± 3.0)
6	512 (± 46)	20.32 (± 4.1)
8	810 (± 59)	32.00 (± 2.8)
10	1300 (± 81)	51.15 (± 2.6)
15	3845 (± 76)	135.00 (± 4.2)
20	5876 (± 58)	189.53 (± 5.1)
25	6352 (± 72)	204.11 (± 6.0)
30	6772 (± 58)	229.00 (± 7.8)

* Data represent average of six replicates.
Figures in the paranthesis represent standard errors.

Table 9 : Standard Growth Curve of *Crotalaria* Suspension Cultures Grown in Synthetic Medium*

Inoculum : 300± 30 mg tissue by fresh weight (dry weight 11.0 mg) in 40 ml of modified Murashige and Skoog medium (Table 1, Chapter II) supplemented with 2.0% sucrose and 2.0 mg/l 2,4-D.

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

Time (days)	Fresh Weight (mg/culture)	Dry Weight (mg/culture)
0	300± 30	10.00 (± 2.1)
2	306 (± 32)	11.46 (± 2.3)
4	361 (± 41)	13.00 (± 4.1)
6	508 (± 31)	18.10 (± 3.8)
8	792 (± 58)	27.12 (± 4.7)
10	1298 (± 62)	48.45 (± 5.1)
15	3546 (± 85)	123.00 (± 5.9)
20	5275 (± 89)	168.08 (± 6.3)
25	5892 (± 72)	183.15 (± 6.0)
30	6256 (± 58)	198.11 (± 4.8)

* Data represent average of six replicates.
Figures in the paranthesis represent standard errors.

Fig. B-5 and B-6. Progressive changes in growth as measured by fresh (Fig. B-5) and dry (Fig. B-6) weights of callus and suspension cultures.

Inoculum : 300± 30 mg tissue by fresh weight on 30 ml (40 ml in case of suspensions) MS medium supplemented with 2.0 mg/l 2,4-D.

Experimental details as given in Table 8 and Table 9.

Fig. B-7 and B-8. Progressive changes in growth in terms of semilog basis (Fig. B-7 fresh wts. ad and Fig. B-8 dry wts.), as calculated from the formula $K = \frac{1}{T} \times \log \frac{X}{Xo} \times Ke$, on standard MS medium.

Experimental details as given in Table 8 and Table 9.

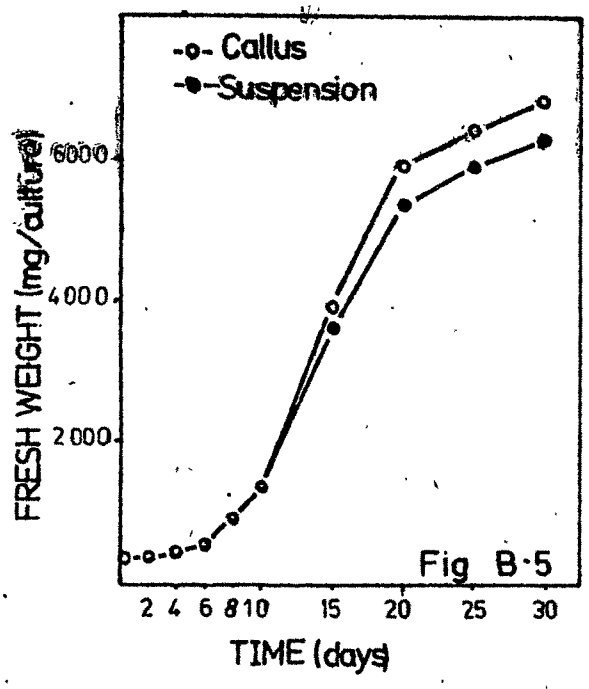


Fig B-5

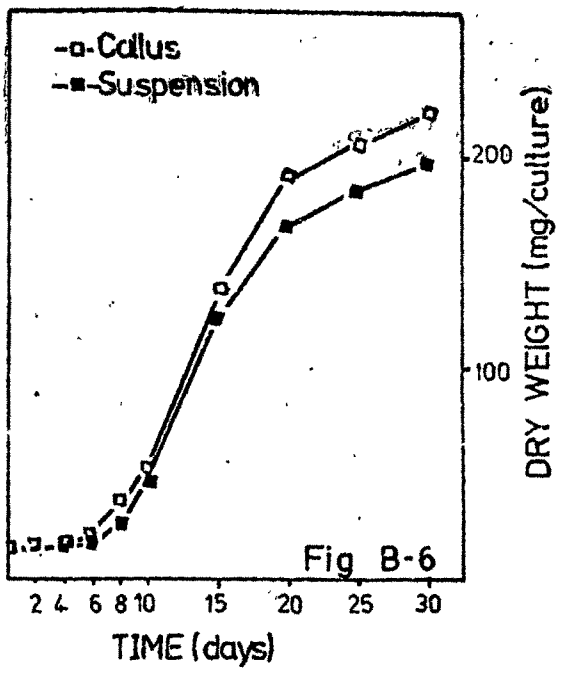


Fig B-6

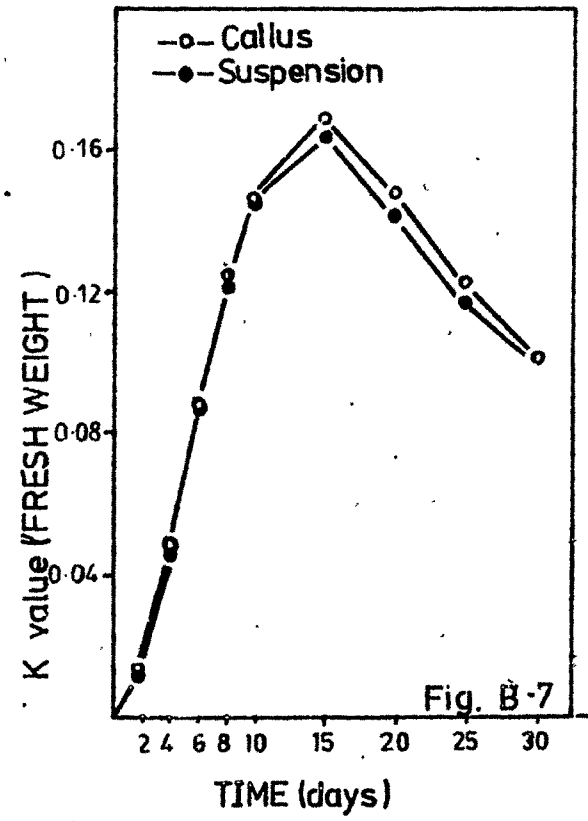


Fig. B-7

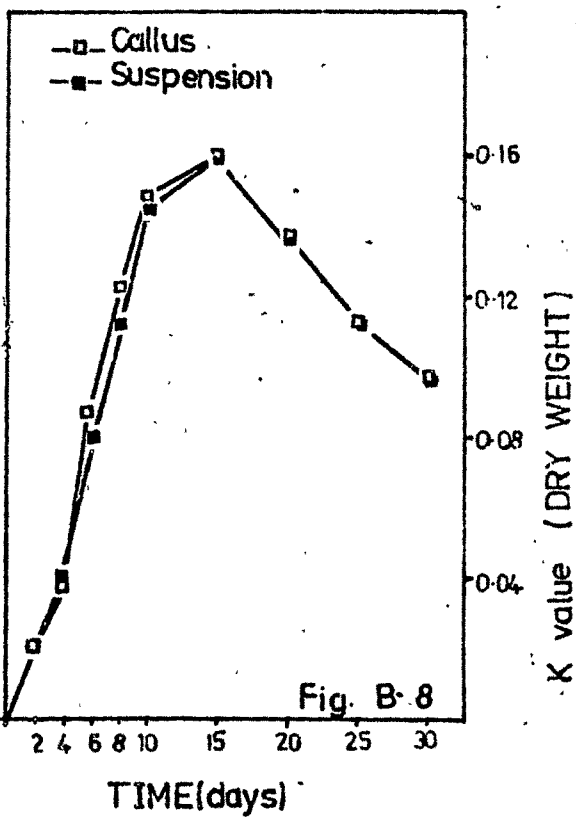


Fig. B-8

suspension cultures. After an initial slow growth for about 4-6 days there was rapid increase in growth upto 20 days. Maximum growth was recorded between 10 to 20 days. During the course of incubation for 30 days, an overall 22.57 fold increase in fresh weight and 20.82 fold increase in dry weight were recorded for callus culture and 20.85 fold increase in fresh weight and 19.81 fold increase in dry weight were recorded for suspension culture.

Progress in growth plotted on semilog basis according to growth kinetics formula, $K = \frac{1}{T} X \log \frac{X}{X_O} X Ke$, described by Price (1970), is presented in Fig. B. 7 and Fig. B-8. After very brief period of initial lag phase, exponential growth continued upto day 10. Decline in semilog values of fresh and dry weights after 15 days indicated slow growth rate from 15 day onwards.

SUMMARY

It is clear from the experiments that the callus which was best initiated on MS medium supplemented with 2.0 mg/l 2,4-D, 2.0 mg/l IAA and 2.0 mg/l kinetin grew

quite satisfactorily when transferred to a medium containing only 2.0 mg/l 2,4-D in addition to 2.0% sucrose.

Of the auxins tested, 2,4-D supported highest growth of the tissue. Kinetin failed to enhance the growth in presence of any of the auxins (i.e. 2,4-D, NAA and IAA). MS medium supplemented with 2.0 mg/l supported maximum growth of the tissue.

When the growth curves of Crotalaria callus and suspension cultures were examined, typical sigmoid curves were observed for both the cultures. After slow growth phase of about 6 days, there was rapid increase in growth upto day 15. However, when the data was plotted on semilog basis, the picture was different. Lag phase lasted only for about 2 days and 2-10 days period constituted exponential growth phase. Further, the growth values recorded for callus cultures were higher than the growth values recorded for suspension cultures.

C. NUTRITIONAL STUDIES ON GROWTH AND POLYPHENOL ACCUMULATION IN *CROTALARIA* CALLUS CULTURES

Growth of plants and their isolated tissue is dependent on the availability of a balanced milieu of nutrient factors. Since carbon and nitrogen sources play vital roles in the tissue growth, a search was conducted for best carbohydrate and nitrogen sources for the growth of *Crotalaria* callus cultures.

In many cases it is observed that the quantitative and qualitative performance of the tissue in forming secondary products is greatly influenced by the cultural conditions and the nutrient status of the medium. Though nitrogen is not directly involved in the production of phenylpropanoid compounds its requirement is absolute. Experiments were conducted to find best nitrogen source for the accumulation of these compounds following the report (Hahlbrock, 1974) that nitrate metabolism of *Glycine max* and *Petroselinum hortense* cell cultures might in some way be related to phenylpropanoid metabolism. Other nutrient factors examined for their ability to support polyphenol production were carbohydrates, L-phenylalanine, L-tyrosine and urea.

Many phenolic compounds inhibit the growth of plants. Experiments were, therefore, also conducted to study the effect of some phenolic acids on growth as well as on the accumulation of phenolic materials.

1. Effect of Different Sugars on Growth and Polyphenol Production in Crotalaria Callus Cultures.

Experiments were designed to find out a suitable carbohydrate source for continuous and rapid growth as well as for enhanced production of phenolic compounds in Crotalaria callus cultures. Callus masses weighing approximately 300± 30 mg tissue by fresh weight were enoculated separately onto 30 ml of agar medium in 150 ml Erlenmeyer flasks containing :-

A. MS medium without sugar,

B. MS medium with 2.0% sucrose,

C. MS medium with 2.0% glucose,

D. MS medium with 2.0% fructose,

E. MS medium with 1.0% glucose + 1.0% fructose,

F. MS medium with 2.0% maltose,

G. MS medium with 2.0% starch, and

H. MS medium with 2.0% mannitol.

The flasks were incubated in a continuously illuminated culture room at a constant temperature of 26± 2°C. A fixed number of replicate flasks of each treatment was harvested after 30 days for the determination of fresh weight, dry weight and total phenolic materials of the tissues as described in Chapter II, Materials and Methods, 5A, 5B and 6A.

The influence of various sugars on growth and accumulation of total phenolic compounds is illustrated in Fig. C-1 and Table 10. Growth was very poor in absence of carbohydrate (medium A). It was maximum on sucrose medium (medium B); while mannitol failed to support growth (medium H). Growth attained when glucose (medium C) and fructose (medium D) were supplied separately was almost similar to that when equimolar mixture of glucose and fructose was added together (medium E). Sucrose supported maximum polyphenol production also. While glucose had slight edge over fructose for polyphenol production, equimolar mixture of both supported almost similar production of phenolic compounds as that of fructose. Though better than starch, maltose did not prove to be an efficient carbohydrate source of energy

Table 10 : Effect of Different sugars on Growth and Polyphenol Accumulation in Callus Cultures of Crotalaria*

Inoculum : 300± 30 mg tissue by fresh weight on 30 ml MS medium supplemented with 2.0 mg/l 2,4-D and :-

(A) without sugar,
(B) 2.0% sucrose,
(C) 2.0% Glucose,
(D) 2.0% Fructose,
(E) 1.0% Glucose + 1.0% Fructose,
(F) 2.0% Maltose,
(G) 2.0% Starch, and
(H) 2.0% Mannitol.

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

Medium	Fresh wt. (mg)	Dry wt. (mg)	Polyphenols	
			g/culture	g/100 mg dry wt.
A	540 (±11)	19 (± 1.2)	38	200
B	6770 (± 58)	229 (± 7.8)	1889	825
C	4314 (± 36)	157 (± 2.8)	863	549
D	3875 (± 52)	147 (± 3.9)	678	461
E	3967 (± 72)	148 (± 5.6)	695	469
F	2336 (± 36)	83 (± 2.2)	350	422
G	1349 (± 29)	44 (± 1.9)	202	459
H	614 (± 10)	22 (± 1.5)	104	473

* Data represent average of six replicates.
Figures in the paranthesis represent standard errors.

Fig. C-1. Effect of different carbohydrates on growth and production of phenolic compounds in *Crotalaria* callus cultures.

Treatments : MS medium without carbohydrate (A), supplemented with 2.0% sucrose (B), 2.0% glucose (C), 2.0% fructose (D), 1.0% glucose + 1.0% fructose (E), 2.0% maltose (F), 2.0% starch (G), 2.0% mannitol (H).

Inoculum : 300 ± 30 mg tissue by fresh weight on 30 ml medium.

Experimental details as given in Table 10.

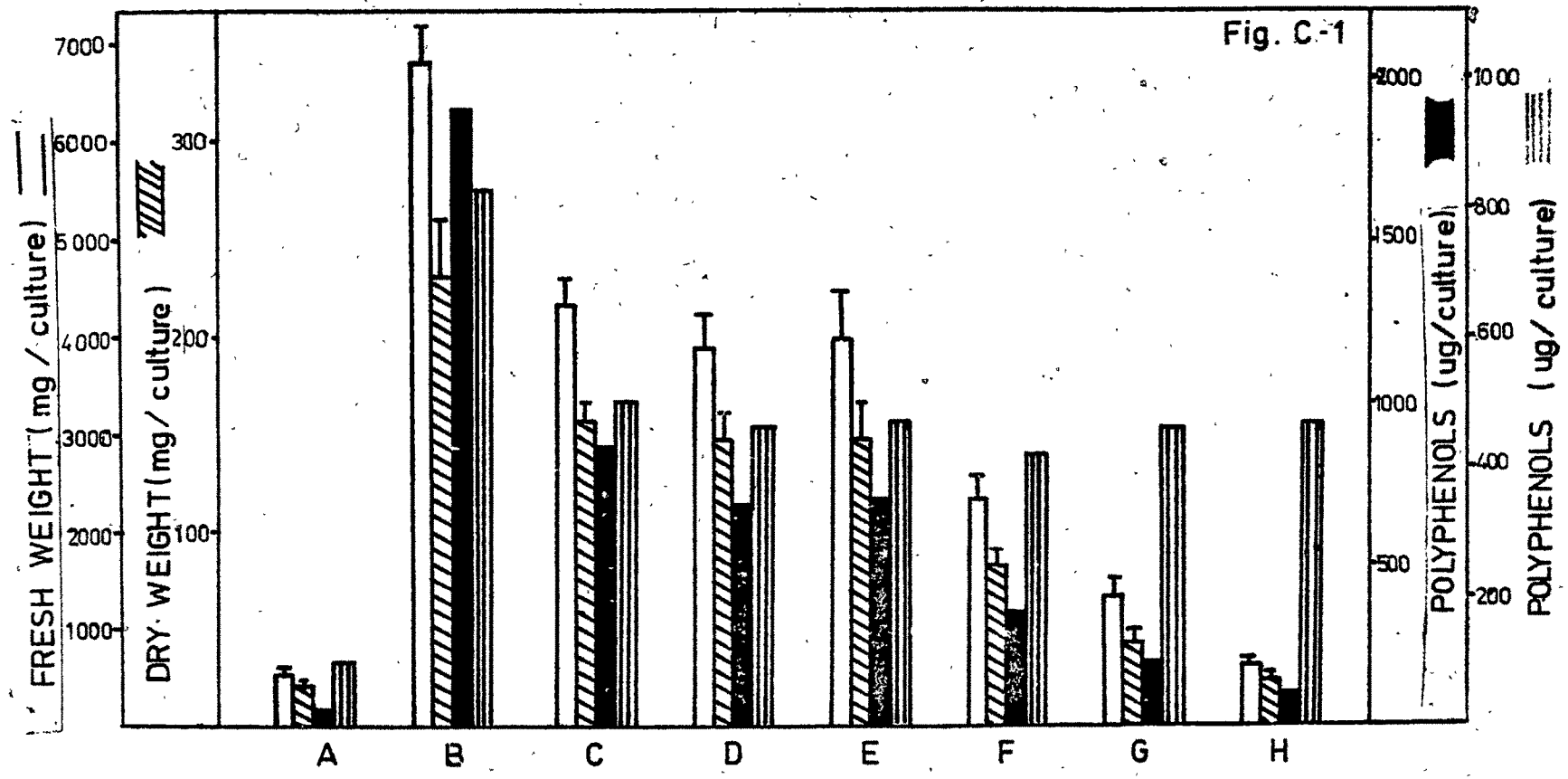


Fig. C-1
FRESH WEIGHT (mg / culture)
DRY WEIGHT (mg / culture)
POLYPHENOLS (ug/culture)
POLYPHENOLS (ug/ culture)
7000
6000
5000
4000
3000
2000
1000
300
200
100
2000
1500
1000
500
1000
800
600
400
200
A
B
C
D
E
F
G
H

for the rapid growth of the cells and also for the production of phenolic materials (per culture). Medium lacking carbohydrate failed to support polyphenol synthesis. On percentage basis also sucrose proved efficient carbon source for polyphenol production. However, other sources of carbohydrate supported polyphenol synthesis in the range of 0.42 to 0.55%.

2. Effect of Nitrogen Sources on Growth and Polyphenol Accumulation in Callus Cultures of Crotalaria.

To determine the ability of Crotalaria callus cultures in utilizing different levels of nitrogen and different sources of nitrogen for the growth of the tissue and for total polyphenol production, weighed tissue masses (300± 30 mg by fresh weight) were separately inoculated onto 30 ml of agar medium containing variables to be tested. The culture vessels were incubated for 30 days in a continuously illuminated culture room at a constant temperature of 26± 2°C.

Experiments carried out on the effect of nitrogen sources on growth and polyphenol production were broadly classified as :-

A. Effect of different levels of nitrogen,

B. Inorganic nitrogen sources,

C. Inorganic nitrogen sources in different combinations,

D. Organic nitrogen sources, and

E. Effect of different concentrations of urea.

A. Effect of different levels of nitrogen

The following nitrogen levels were tested to study the effect on growth and polyphenol production. The nitrogen contents of vitamins are not considered.

1. 0.0 mg/l nitrogen (MS medium without nitrates),
2. 52 mg/l nitrogen (White's medium with 2.0 mg/l 2,4-D and 10% coconut milk),
3. 420 mg/l nitrogen (MS medium with 825 mg/l NH_4NO_3 and 950 mg/l KNO_3),
4. 840 mg/l nitrogen (standard MS medium), and
5. 1260 mg/l nitrogen (MS medium with 2475 mg/l NH_4NO_3 and 2850 mg/l KNO_3).

Polyphenol accumulation and growth responses determined after incubation period of 4 weeks are presented in Fig. C-2 and Table 11. Standard MS medium supported maximum growth and polyphenol synthesis; while White's medium supported poor growth as compared to any of the MS media tested with nitrogen the polyphenol production was more than sub- and supra-optimal nitrogen containing MS media. Also, in the range of N level in MS media, suboptimal nitrogen containing medium was found better than supraoptimal nitrogen containing medium both for the growth of the cells and for the production of phenolic materials. Medium without nitrogen source failed to support growth and polyphenol production. When the accumulation of phenolic compounds was observed on unit basis, it became clear that the ability of cells to accumulate polyphenols increased on the medium containing less nitrogen. Thus, White's medium containing only 52 mg/l nitrogen supported 1.1% of phenolic compounds and MS medium with highest nitrogen content tested (1260 mg/l) supported only 0.767% of phenolic materials. Standard MS medium (840 mg/l nitrogen) supported 0.826% and sub-optimal

Table 11 : Effect of Different Levels of Nitrogen Source on Growth and Polyphenol Production in Callus Cultures of Crotalaria *

Inoculum : 300± 30 mg tissue by fresh weight on 30 ml agar medium containing 2.0% sucrose, 2.0 mg/l 2,4-D and :-

A. MS medium without nitrogen source,

B. White's medium with 10% coconut milk,

C. MS medium with 825 mg/l NH_4NO_3 and 950 mg/l KNO_3,

D. MS medium with 1650 mg/l NH_4NO_3 and 1900 mg/l KNO_3, and

E. MS medium with 2475 mg/l NH_4NO_3 and 2850 mg/l KNO_3.

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

Medium	Fresh wt. (mg)	Dry wt. (mg)	Polyphenols	
			g/culture	g/100 mg dry wt.
A	545 (± 12)	29 (± 1.2)	354	1221
B	3152 (± 43)	146 (± 5.2)	1606	1100
C	4382 (± 72)	158 (±11.2)	1452	919
D	6772 (± 58)	229 (± 7.8)	1889	825
E	3742 (± 63)	138 (± 8.0)	1059	767

* Data represent average of six replicates.
Figures in the paranthesis represent standard errors.

Table 12 : Effect of Nitrogen Source on Growth and Polyphenol Production in Callus Cultures of Crotalaria*

Inorganic Nitrogen Sources.

Inoculum : 300± 30 mg tissue by fresh weight on 30 ml MS medium supplemented with 2.0% sucrose, 2.0 mg/l 2,4-D and equimolar solution (in terms of nitrogen content, 840 mg/l) of :

1. Potassium nitrate,
2. Ammonium nitrate,
3. Sodium nitrate,
4. Potassium nitrite,
5. Ammonium nitrite, and
6. Sodium nitrite.

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

Medium	Fresh wt. (mg)	Dry wt. (mg)	Polyphenols	
			g/culture	g/100 mg dry wt.
1	6128 (± 48)	212 (± 1.8)	1060	500
2	4464 (± 29)	159 (± 2.0)	1431	900
3	5810 (± 33)	201 (± 2.2)	1206	600
4	728 (± 21)	32 (± 0.8)	96	300
5	590 (± 26)	25 (± 0.9)	118	472
6	685 (± 18)	27 (± 1.0)	103	381

* Data represent average of six replicates.
Figures in the paranthesis represent standard errors.

Fig. C-2. Growth and accumulation of phenolic compounds in callus tissues as influenced by various concentrations of nitrogen in the medium.

Treatments : (A) MS medium without nitrogen,
(B) 52 mg/l nitrogen (White's medium),
(C) 420 mg/l nitrogen (MS medium)
(D) 840 mg/l nitrogen (")
and (E) 1260 mg/l nitrogen (")

Inoculum : 300 ± 30 mg tissue by fresh weight on 30 ml medium.

Experimental details as given in Table 11.

Fig. C-3. Effect of various nitrogen sources on growth and polyphenol production in Crotalaria callus cultures.

Treatments : (1) KNO_3, (2) NH_4NO_3, (3) $NaNO_3$, (4) KNO_2, (5) NH_4NO_2 and (6) $NaNO_2$.

Experimental details as given in Table 12.

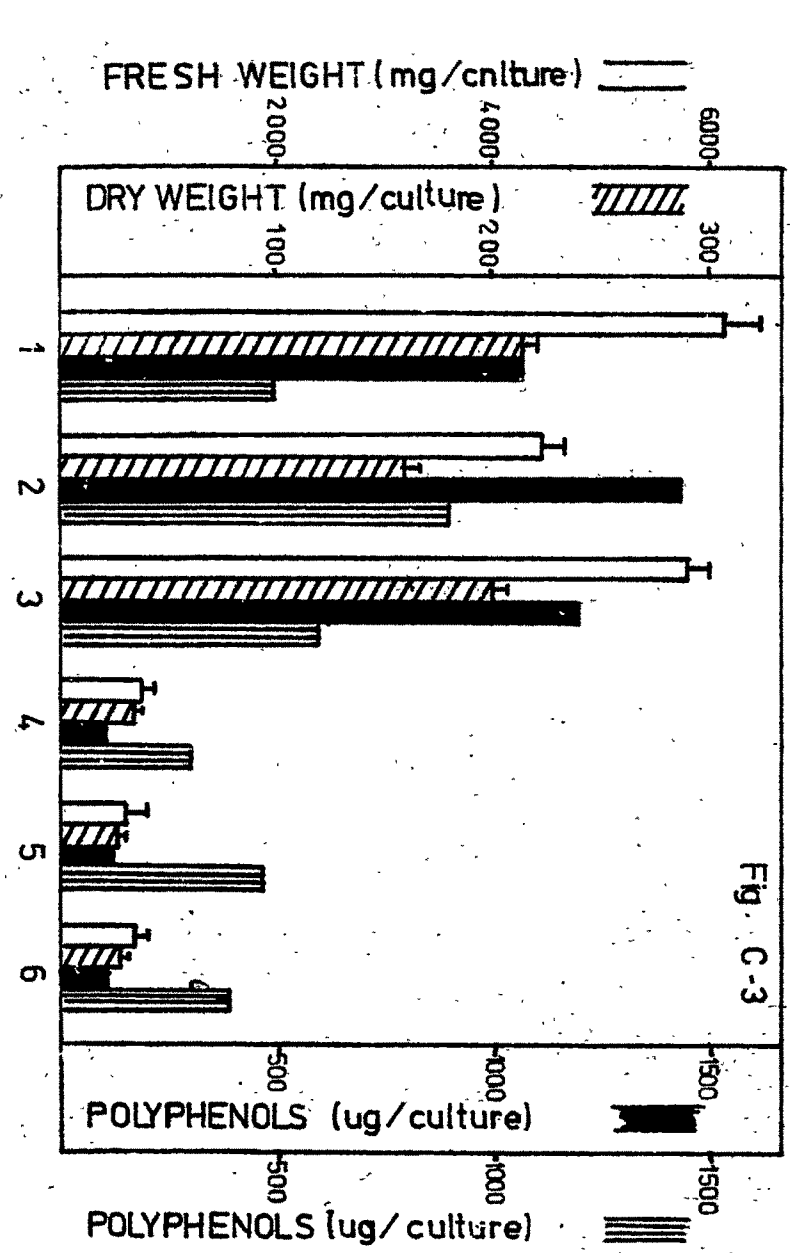

FRESH WEIGHT (mg/culture)
2000
4000
6000
DRY WEIGHT (mg/culture)
100
200
300
1
2
3
4
5
6
500
1000
1500
POLYPHENOLS (ug/culture)
500
1000
1500
POLYPHENOLS (ug/culture)

Fig. C-3

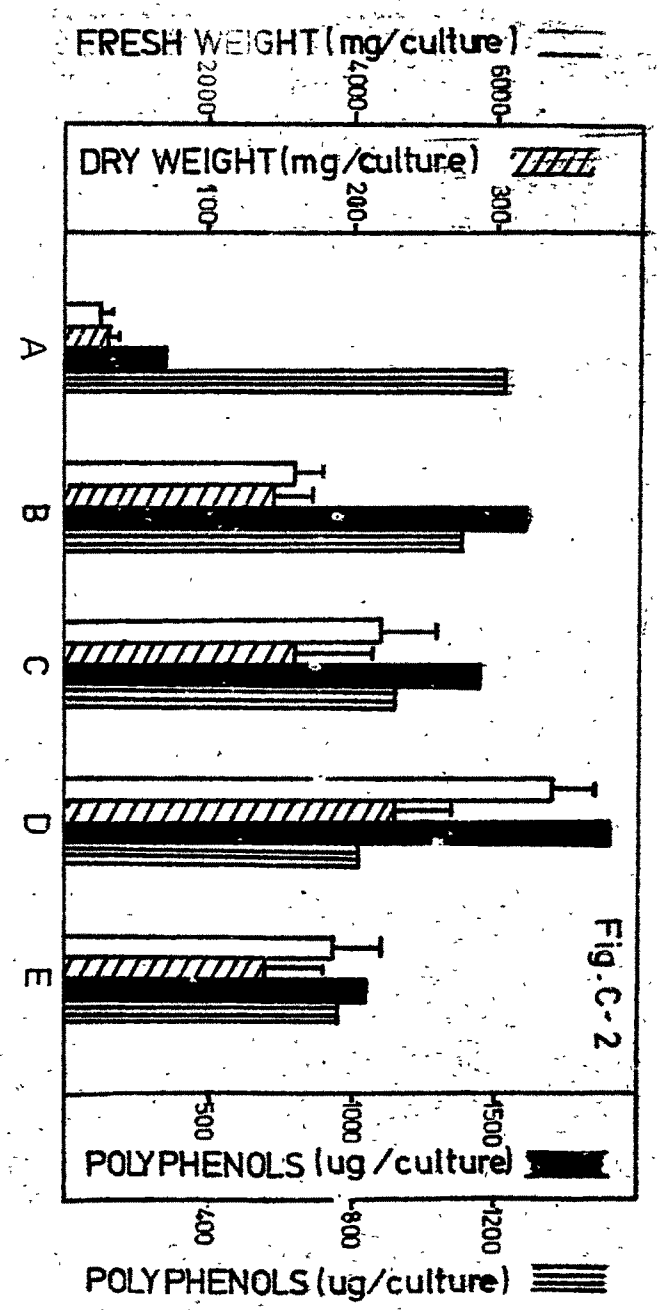

FRESH WEIGHT (mg/culture)
2000
4000
6000
DRY WEIGHT (mg/culture)
100
200
300
A
B
C
D
E
500
1000
1500
POLYPHENOLS (ug/culture)
400
800
1200
POLYPHENOLS (ug/culture)

Fig. C-2

nitrogen containing medium (420 mg/l) supported 0.919% of phenolic compounds. The maximum polyphenol accumulation on unit basis (1.22%) was registered with nitrogen-free MS medium.

B. Inorganic nitrogen sources

Different nitrate and nitrite sources were tested for their ability to support growth and polyphenol production. Equimolar solutions in terms of their nitrogen content were added to the MS medium and tissue masses weighing 300± 30 mg by fresh weight were separately inoculated onto each medium. After 30 days of incubation in light at 26± 2°C, a fixed number of replicate flasks of each treatment was harvested for the determination of fresh weight, dry weight and total phenolic compounds of the tissues as described in Chapter II, Materials and Methods, 5A, 5B and 6A.

The effect of different nitrate and nitrite sources on growth and polyphenols accumulation is shown in Fig. C-3 and Table 12. Clearly, nitrites proved very poor source for the growth of the cells and also for the production of phenolic materials. Among the nitrate

sources, potassium nitrate supported maximum growth and ammonium nitrate proved the poorest source for the growth of the cells. However, polyphenol production was maximum in the tissues grown on ammonium nitrate containing medium. Though better than potassium nitrate, sodium nitrate did not prove good source of nitrogen for the production of phenolic compounds. On the unit basis (polyphenols/100 mg dry wt.), ammonium nitrate supported 0.9% whereas sodium nitrate and potassium nitrate 0.6% and 0.5 of phenolic materials respectively. Among the nitrites also, maximum polyphenols was registered in the tissues grown on the ammonium nitrite medium, but maximum growth was recorded in the tissues grown on potassium nitrite medium.

C. Inorganic nitrogen sources in different combinations

To find out the most suitable combination of nitrogen sources for the growth and polyphenol production MS medium (without NO_3) was supplemented with equimolar solutions (in terms of nitrogen content, 840 mg/l) of the following combinations :

1. NH_4NO_3 (1650 mg/l) and KNO_3 (1900 mg/l),

2. KNO_3 (1900 mg/l) and $NaNO_3$ (3500 mg/l),
3. NH_4NO_3 (1650 mg/l) and $NaNO_3$ (1600 mg/l),
4. KNO_3 (1900 mg/l) and $NaNO_2$ (2842 mg/l),
5. KNO_3 (1900 mg/l) and NH_4NO_2 (1319 mg/l),
6. NH_4NO_3 (1650 mg/l) and KNO_2 (1598 mg/l), and
7. NH_4NO_3 (1650 mg/l) and $NaNO_2$ (1295 mg/l).

Growth responses and the accumulation of phenolic compounds as influenced by various combinations of nitrogen sources after an incubation period of 4 weeks in light at $26 \pm 2^{o}C$ are presented in Fig. C-4 and Table 13.

The tissues cultured on the medium containing ammonium nitrate and potassium nitrate as nitrogen sources showed maximum growth as determined by two cultural parameters (i.e. fresh and dry weights) and also maximum polyphenol production. The combination of K-nitrate with Na-nitrate and Na-nitrite proved to be superior to the combination of NH_4-nitrate with Na-nitrate and Na-nitrite. However, ammonium nitrate-sodium nitrite combination supported more polyphenols, per culture as

Table 13 : Effect of Nitrogen Source on Growth and Polyphenol Production in *Crotalaria* Callus Cultures*

Inorganic Nitrogen Sources in Different Combinations.

Inoculum : 300± 30 mg tissue by fresh weight on 30 ml MS medium supplemented with 2.0% sucrose, 2.0 mg/l 2,4-D and equimolar mixture (in terms of nitrogen content, 840 mg/l) of :-

1. NH_4NO_3 and KNO_3 ,
2. KNO_3 and $NaNO_3$,
3. NH_4NO_3 and $NaNO_3$,
4. KNO_3 and $NaNO_2$,
5. KNO_3 and NH_4NO_2,
6. NH_4NO_3 and KNO_2, and
7. NH_4NO_3 and $NaNO_2$.

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

Medium	Fresh wt. (mg)	Dry wt. (mg)	Polyphenols	
			g/culture	g/100 mg dry wt.
1	6772 (± 58)	229 (± 7.8)	1889	825
2	4483 (± 62)	149 (± 5.3)	596	400
3	3026 (± 36)	94 (± 6.2)	282	300
4	2853 (± 42)	89 (± 7.1)	356	400
5	2231 (± 53)	72 (± 5.1)	272	378
6	1873 (± 38)	61 (± 4.3)	366	600
7	2051 (± 23)	64 (± 6.5)	384	600

* Data represent average of six replicates.
Figures in the paranthesis represent standard errors.

Fig. C-4. Effect of nitrogen sources, added in different combinations, on growth and polyphenol accumulation in callus cultures.

Inoculum : 300± 30 mg tissue by fresh weight on 30 ml MS medium supplemented with 2.0 mg/l 2,4-D and equimolar mixture (in terms of nitrogen content, 840 mg/l) of :

(1) NH_4NO_3 and KNO_3, (2) KNO_3 and $NaNO_3$, (3) NH_4NO_3 and $NaNO_3$, (4) KNO_3 and $NaNO_2$, (5) KNO_3 and NH_4NO_2, (6) NH_4NO_3 and KNO_2, and (7) NH_4NO_3 and $NaNO_2$.

Other experimental details as given in Table 13.

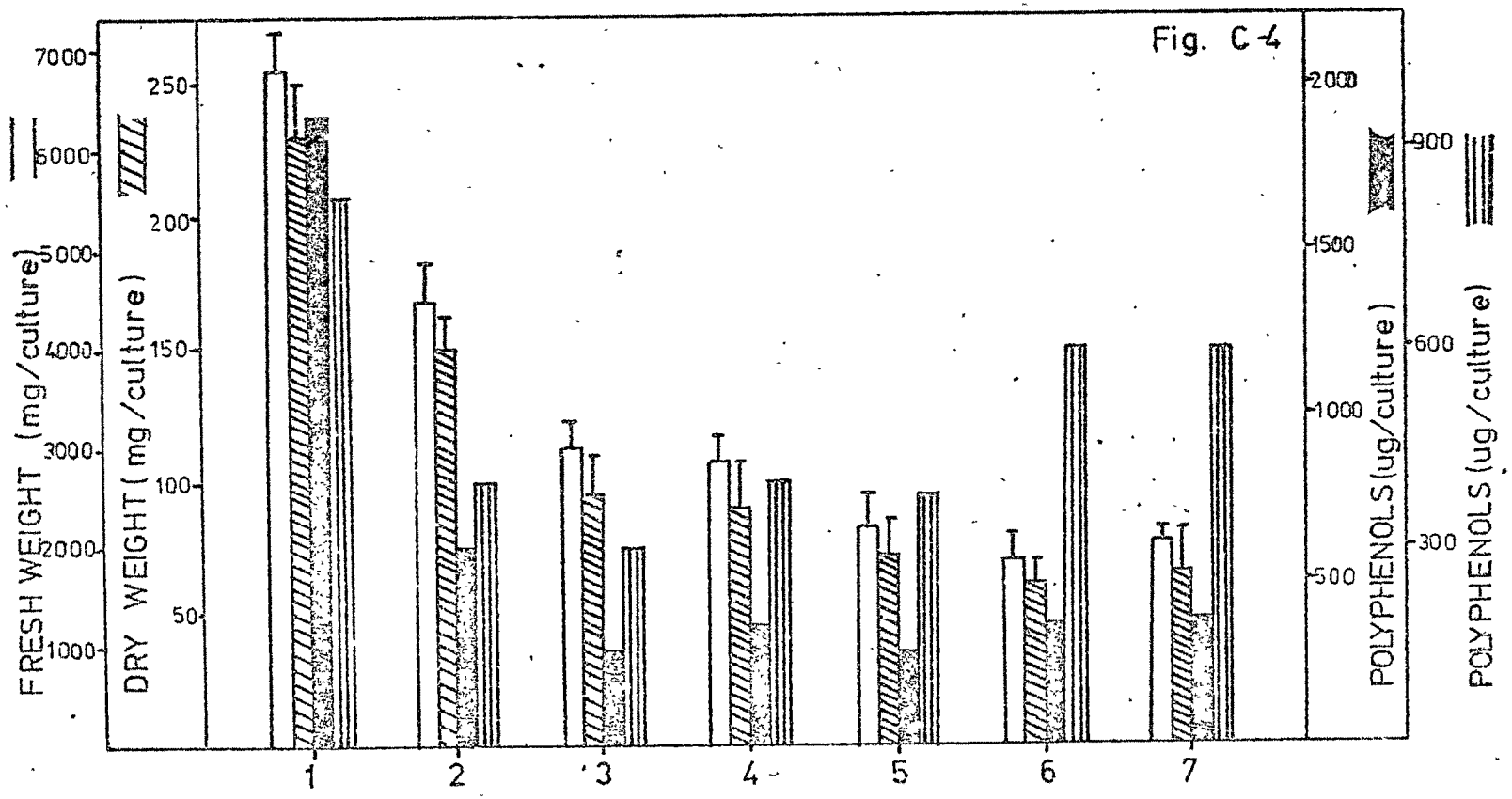


Fig. C-4
FRESH WEIGHT (mg/culture)
DRY WEIGHT (mg/culture)
POLYPHENOLS (ug/culture)
POLYPHENOLS (ug/culture)
7000
5000
4000
3000
2000
1000
250
200
150
100
50
2000
1500
1000
500
900
600
300
1
2
3
4
5
6
7

well as on unit basis, than ammonium nitrate-sodium nitrate combination. On the other hand, the nitrate-nitrate combination of potassium and sodium was superior to that of K-nitrate-Na-nitrite combination. The implications of the interactions between these nitrogen sources and their effects when used singly (2 B) are elaborated and discussed in Chapter IV.

The results also showed that incorporation of ammonium salt into the nutritive medium resulted into enhanced accumulation of phenolic compounds on one hand, and suppression of growth on the other; indicating different optimal N-source for the two processes. However, a balanced supply of potassium and ammonium nitrates not only resulted into maximum polyphenol production but also supported maximum growth.

D. Organic nitrogen sources

To determine the ability of callus cultures of Crotalaria in utilizing different sources of organic nitrogen for the growth of the cells and for total polyphenol production, tissue masses weighing approximately 300± 30 mg by fresh weight were separately

inoculated onto 30 ml MS medium (without nitrates) in Erlenmeyer flasks supplemented with the following sources of organic nitrogen :-

1. Urea (1.81 g/l),
2. Casein hydrolysate (10 mg/l),
3. Yeast extract (2.5 g/l),
4. Yeast extract (5 g/l), and
5. Yeast extract (10 g/l).

Polyphenol content and growth responses of callus cultures to various sources of organic nitrogen after incubation for 4 weeks in light at $26 \pm 2^{\circ}C$ are illustrated in Fig. C-5 and Table 14.

The results clearly showed that urea failed to support growth and phenolic production. Maximum growth and polyphenol accumulation was recorded in the tissues grown on yeast extract (5 g/l) medium. However, growth was suppressed at supraoptimal level of yeast extract. Casein hydrolysate, as sole organic nitrogen source, also proved quite satisfactorily for growth as well as for polyphenol production. Increased yeast extract

Table 14 : Effect of Nitrogen Source on Growth and Polyphenol Production in *Crotalaria* Callus Cultures*

Organic Nitrogen Sources.

Inoculum : 300 ± 30 mg tissue by fresh weight on 30 ml MS medium (without NO_3) supplemented with 2.0% sucrose, 2.0 mg/l 2,4-D and :-

1. Urea (1810 mg/l),
2. Casein hydrolysate (1 g/l),
3. Yeast extract (2.5 g/l),
4. Yeast extract (5 g/l), and
5. Yeast extract 10 g/l).

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

Medium	Fresh wt. (mg)	Dry wt. (mg)	Polyphenols	
			g/culture	g/100 mg dry wt.
1	451 (±12)	16 (± 5.2)	24	150
2	6608 (± 71)	223 (± 8.9)	1784	800
3	4562 (± 38)	169 (± 7.2)	1690	1000
4	6996 (± 67)	248 (± 9.1)	2976	1200
5	3646 (± 52)	136 (± 5.6)	1768	1300

* Data represent average of six replicates.

Figures in the paranthesis represent standard errors.

Table 15 : Effect of Urea on Growth and Polyphenol Accumulation in Crotalaria Callus Cultures*

Inoculum : 300± 30 mg tissue by fresh weight on 30 ml MS medium supplemented with 2.0% sucrose, 2.0 mg/l 2,4-D and :-

A. without Urea,

B. 100 mg/l Urea,

C. 250 mg/l Urea, and

D. 500 mg/l Urea.

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

Medium	Fresh wt. (mg)	Dry wt. (mg)	Polyphenols	
			g/culture	g/100 mg dry wt.
A	6772 (± 58)	229 (± 7.8)	1889	825
B	4360 (± 41)	162 (± 6.7)	810	500
C	3788 (± 32)	138 (± 5.1)	552	400
D	523 (± 23)	22 (± 4.2)	66	300

* Data represent average of six replicates.

Figures in the paranthesis represent standard errors.

Fig. C-5. Growth and accumulation of phenolic materials as influenced by various organic nitrogen sources.

Treatments : Supplemented to MS medium (inorganic nitrogen omitted) :

(1) Urea (1810 mg/l),
(2) Casein hydrolysate (10 g/l),
(3) Yeast extract (2.5 g/l),
(4) Yeast extract (5 g/l), and
(5) Yeast extract (10 g/l).

Inoculum : 300± 30 mg tissue by fresh weight on 30 ml MS medium.

Experimental details as given in Table 14.

Fig. C-6. Effect of different concentrations of Urea on growth and polyphenol production in Crotalaria callus tissues.

Treatments : (A) without Urea,
(B) 100 mg/l Urea,
(C) 250 mg/l Urea, and
(D) 500 mg/l Urea.

Inoculum : 300± 30 mg tissue by fresh weight on 30 ml MS medium.

Experimental details as given in Table 15.

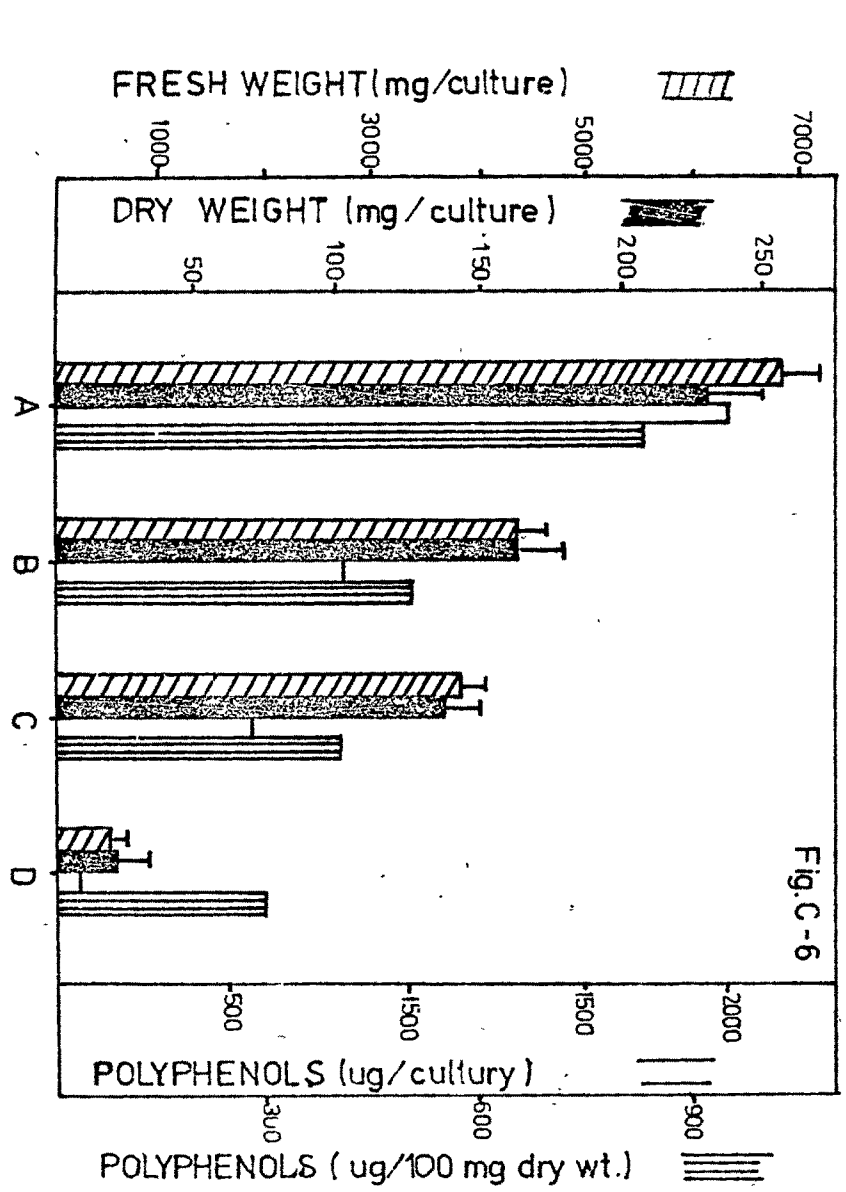

FRESH WEIGHT (mg/culture)
1000
3000
5000
7000
DRY WEIGHT (mg/culture)
50
100
150
200
250
A
B
C
D
500
1000
1500
2000
POLYPHENOLS (ug/cultury)
300
600
900
POLYPHENOLS (ug/100 mg dry wt.)

Fig. C-6

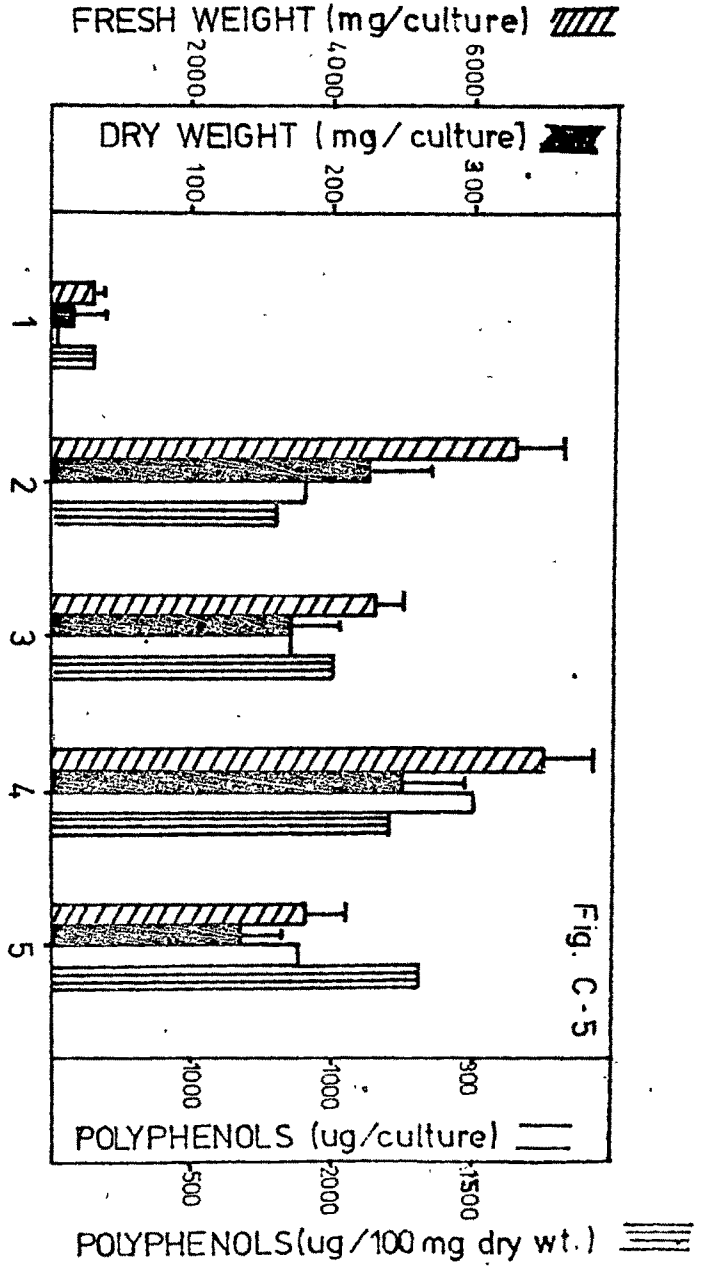

FRESH WEIGHT (mg/culture)
2000
4000
6000
DRY WEIGHT (mg/culture)
100
200
300
1
2
3
4
5
1000
1000
900
POLYPHENOLS (ug/culture)
500
2000
1500
POLYPHENOLS (ug/100 mg dry wt.)

Fig. C-5

concentration of the medium enhanced the production of phenolic compounds on unit basis. Thus, 2500mg/l yeast extract supported 1.0% of phenolic materials whereas 10000mg/l yeast extract supported 1.3% of phenolic compounds, but with markedly adverse effect on tissue growth.

E. Effect of Different concentrations of urea

To examine the ability of urea to support growth and polyphenol accumulation, weighed tissue masses (300± 30 mg by fresh weights) were separately inoculated onto 30 ml MS medium (without NO_3) supplemented with 2.0% sucrose, 2.0 mg/l 2,4-D and the following concentrations of urea :

A. 0.0 mg/l urea,

B. 100 mg/l urea,

C. 250 mg/l urea, and

D. 500 mg/l urea.

The culture vessels were incubated for 30 days in light at 26± 2°C. A fixed number of replicates was

harvested to determine the fresh weight, dry weight and polyphenols as described in Chapter II, Materials and Methods, 5A, 5B and 6A.

Fig. C-6 and Table 15 illustrate the influence of different concentrations of urea on growth and polyphenol production. The results clearly indicated that there was marked inhibitory effect of urea on growth as well as on polyphenol production, the inhibitory effect was very clear at highest level of urea tested (500 mg/l). Also, the ability of cells to accumulate phenolic compounds decreased with increasing levels of urea. 0.826% of phenolic materials was registered in the tissues grown on urea-free control medium whereas 0.5%, 0.4% and 0.3% of phenolic compounds were recorded in the tissues grown on medium containing 100 mg/l, 250 mg/l and 500 mg/l urea respectively.

3. Effect of L-phenylalanine and L-tyrosine on Growth and Polyphenol Synthesis in Callus Cultures of Crotalaria

The experiment described below was conducted to

study the effect of L-phenylalanine and L-tyrosine on growth and production of phenolic compounds in Crotalaria callus cultures (background explained in Chapter I, Introduction).

The MS medium (Table 1, Chapter II) was supplemented with 2.0% sucrose, 2.0 mg/l 2,4-D and the following concentrations of L-phenylalanine or L-tyrosine : 0.0, 0.05, 0.1 and 0.2%. The concentrations tested in combinations were : 0.05% L-phenylalanine + 0.05% L-tyrosine, and 0.1% L-phenylalanine + 0.1% L-tyrosine. Tissues masses weighing 300± 30 mg by fresh weight were inoculated separately to 30 ml medium. The culture flasks were incubated under uniform conditions of light and temperature and a fixed number of replicates was harvested, after 30 days for estimating accumulation of total polyphenols and for growth determination.

The data presented in Fig. C-7 and Table 16 clearly showed that the addition of L-phenylalanine did not enhance the production of phenolic compounds when compared to the control. At all the levels of L-phenylalanine tested, there was pronounced inhibition of growth and polyphenol production. However, on unit

Table 16 : Effect of L-Phenylalanine and L-Tyrosine on Growth and Polyphenol Synthesis in Callus Cultures of Crotalaria*

Inoculum : 300± 30 mg tissue by fresh weight on 30 ml MS medium with :-

A. No L-Phenylalanine and L-Tyrosine (control),
B. 0.05% L-Phenylalanine,
C. 0.1% L-Phenylalanine,
D. 0.2% L-Phenylalanine,
E. 0.05% L-Tyrosine,
F. 0.1% L-Tyrosine,
G. 0.2% L-Tyrosine,
H. 0.05% L-Phenylalanine + 0.05% L-Tyrosine, and
I. 0.1% L-Phenylalanine + 0.1% L-Tyrosine.

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

Medium	Fresh wt. (mg)	Dry wt. (mg)	Polyphenols	
			g/culture	g/100 mg dry wt.
A	6772 (± 58)	229 (± 7.8)	1892	826
B	856 (± 42)	29 (± 6.0)	319	1100
C	898 (± 31)	32 (± 3.0)	480	1500
D	575 (± 20)	20 (± 1.8)	250	1250
E	961 (± 18)	34 (± 2.6)	663	1950
F	3440 (± 63)	113 (± 8.0)	4068	3600
G	1621 (± 26)	53 (± 5.6)	1669	3150
H	1251 (± 21)	41 (± 4.3)	645	1575
I	3252 (± 46)	103 (± 8.7)	2292	2225

* Data represent average of six replicates.
Figures in the paranthesis represent standard errors.

Fig. C-7. Effect of L-phenylalanine and L-tyrosine on growth and accumulation of phenolic compounds in Crotalaria callus cultures.

Treatments : (A) No L-phenylalanine and L-tyrosine (control),
(B) 0.05% L-phenylalanine,
(C) 0.1% L-phenylalanine,
(D) 0.2% L-phenylalanine,
(E) 0.05% L-tyrosine,
(F) 0.1% L-tyrosine,
(G) 0.2% L-tyrosine,
(H) 0.05% L-phenylalanine + 0.05% L-tyrosine, and
(I) 0.1% L-phenylalanine + 0.1% L-tyrosine.

Inoculum : 300± 30 mg tissue by fresh weight on 30 ml medium.

Experimental details as given in Table 16.

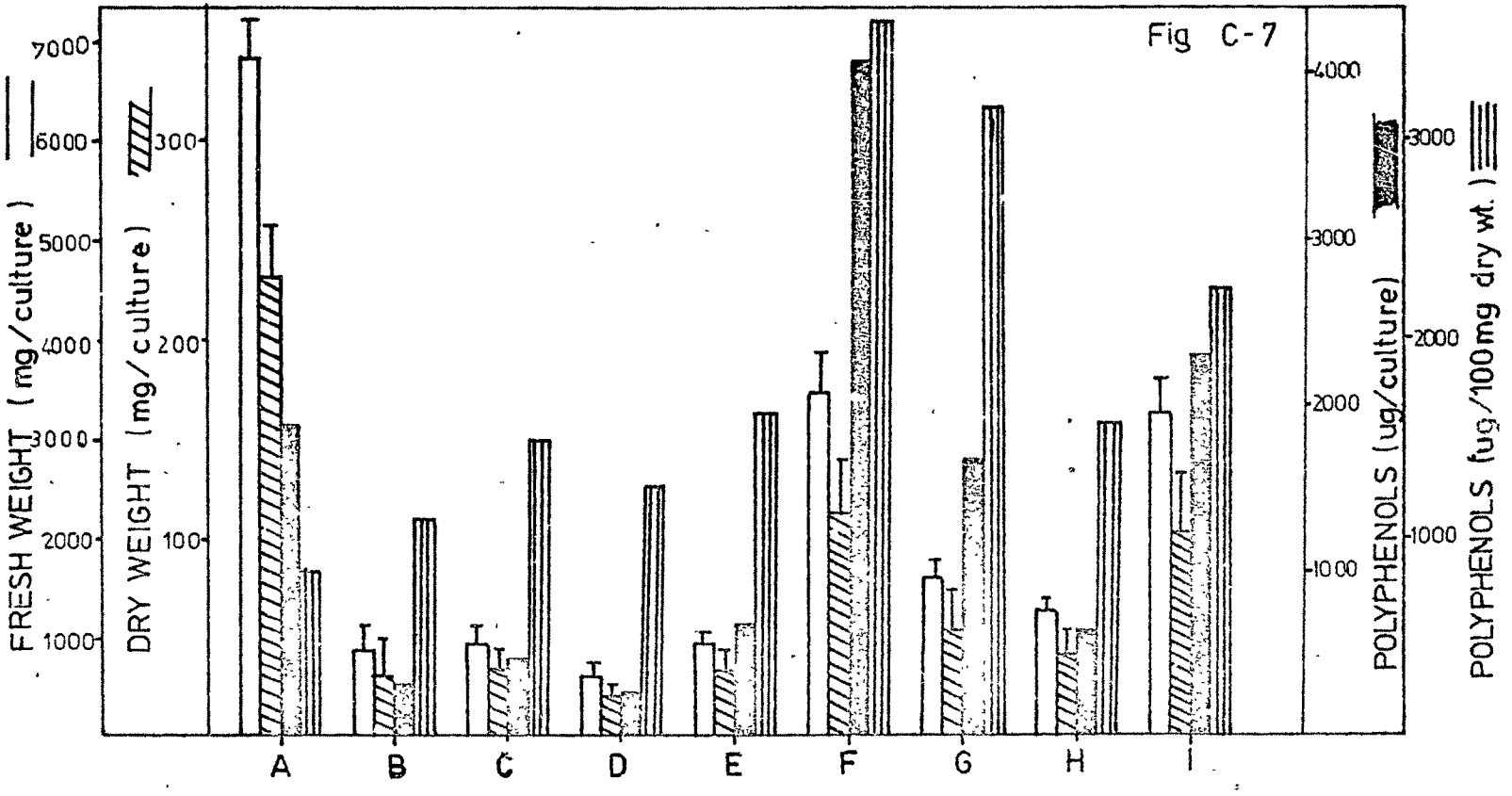


Fig C-7
FRESH WEIGHT (mg/culture)
DRY WEIGHT (mg/culture)
POLYPHENOLS (ug/culture)
POLYPHENOLS (ug/100mg dry wt.)
7000
6000
5000
4000
3000
2000
1000
300
200
100
4000
3000
2000
1000
3000
2000
A
B
C
D
E
F
G
H
I

basis, L-phenylalanine supported production of more phenolic materials than the control. 1.5% of phenolics was registered in tissues grown on 0.1% L-phenylalanine medium as compared to 0.826% recorded for the control medium. L-tyrosine, on the other hand, supported more growth and polyphenol production than L-phenylalanine. Highest (3.6%) accumulation of phenolic compounds was observed in tissues grown on 0.1% L-tyrosine containing medium. Thus, though L-tyrosine suppressed growth, it was highly stimulatory to the synthesis of phenolic compounds. When L-phenylalanine and L-tyrosine were added together, a soft of antagonism was observed. Tyrosine appreciably relieved the pronounced reduction in growth and polyphenol production caused by phenylalanine but its own promoting effect when used alone was much less in combination with phenylalanine. A combination of 0.1% L-phenylalanine and L-tyrosine was found better than 0.1% L-phenylalanine but poorer when compared to 0.1% tyrosine, both for the growth of the cells and also for the production of polyphenols.

4. Effect of Phenolic Acids on Growth and Polyphenol Production in *Crotalaria* Callus Cultures

Six phenolic acids were examined in presence and

absence of 2,4-D at 0.1, 1.0 and 10.0 mg/l concentrations for their ability to support growth and polyphenol accumulation. 300± 30 mg tissue by fresh weight were separately inoculated on 30 ml MS medium supplemented with the following six phenolic acids with or without 2,4-D :

1. p-Hydroxybenzoic acid,
2. Chlorogenic acid,
3. Caffeic acid,
4. Fefulic acid,
5. Cinnamic acid, and
6. p-Coumaric acid.

The culture flasks were incubated at 26± 2°C in light for 30 days. A fixed number of replicate flasks was harvested to determine fresh weight, dry weight and total phenolic compounds.

The influence of various phenolic acids on growth and polyphenol production in presence of 2,4-D is illustrated in Figs. C-8 to C-13 and Table 17. All the phenolic acids inhibited growth at all the concentrations

tested, the higher levels being more inhibitory. Cinnamic acid at 10.0 mg/l concentration inhibited 85% of growth by fresh weight and 81% by dry weight. The lowest inhibition of growth (2.0% by fresh weight and 0.5% by dry weight) was observed in the tissues grown on 0.1 mg/l p-hydroxybenzoic acid medium. At low concentration (0.1 mg/l) p-coumaric acid was found more effective growth inhibitor than the other phenolic acids. However, the inhibitory effect on growth with increasing concentrations of p-coumaric acid and also of caffeic and ferulic acids was less marked than that of p-hydroxybenzoic, chlorogenic and cinnamic acid. Production of phenolic compounds was also inhibited by all the phenolic acids at all the concentrations tested. Maximum inhibition was recorded in the tissues grown on cinnamic acid medium and minimum inhibition was observed with p-hydroxybenzoic acid medium, as was the case with tissue growth also. On unit basis, maximum production of phenolic compounds as supported by phenolic acid containing medium was 0.65% (by 0.1 mg/l p-hydroxybenzoic acid and 1.0 mg/l caffeic acid) as against 0.825% of control medium (MS medium with 2.0 mg/l 2,4-D).

Fig. C-8 to Fig. C-13. Effect of different phenolic acids at 0.1, 1.0 and 10.0 mg/l concentrations in presence of 2.0 mg/l 2,4-D on growth and accumulation of phenolic compounds in callus cultures.

Inoculum : 300± 30 mg tissue by fresh weight on 30 ml MS medium.

Other experimental details as given in Table 17.

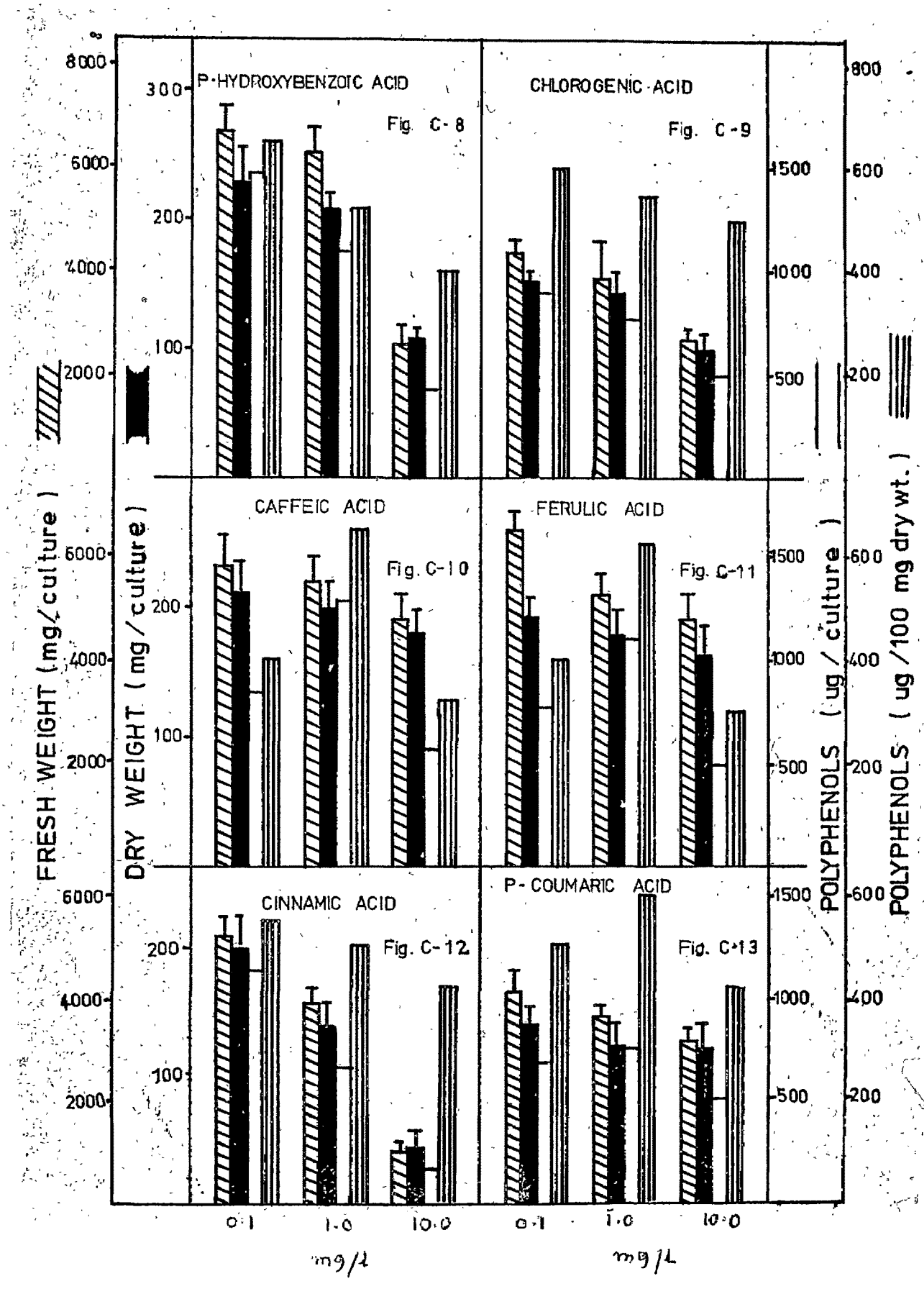

P-HYDROXYBENZOIC ACID
Fig. C-8
CHLOROGENIC ACID
Fig. C-9
CAFFEIC ACID
Fig. C-10
FERULIC ACID
Fig. C-11
CINNAMIC ACID
Fig. C-12
P-COUMARIC ACID
Fig. C-13
FRESH WEIGHT (mg/culture)
DRY WEIGHT (mg/culture)
POLYPHENOLS (ug/culture)
POLYPHENOLS (ug/100 mg dry wt.)
0·1
1·0
10·0
mg/l

Table 17 : Effect of Phenolic Acids in Presence of 2,4-D on Growth and Polyphenol Production in Crotalaria Callus Cultures *

Inoculum : 3000± 30 mg tissue by fresh weight on 30 ml MS medium supplemented with 2.0% sucrose, 2.0 mg/l 2,4-D and phenolic acids as given below.

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

Phenolic Acid	Concentration (mg/l)	Fresh Wt. (mg)	Dry Wt. (mg)	Polyphenols	
				ug/culture	ug/100 mg dry wt.
p-Hydroxybenzoic acid	0.1	6652 (± 48)	228 (± 6.5)	1482	650
"	1.0	6261 (± 52)	208 (± 2.8)	1092	525
"	10.0	2567 (± 38)	107 (± 1.9)	428	400
Chlorogenic acid	0.1	4397 (± 21)	152 (± 2.2)	912	600
"	1.0	3913 (± 70)	143 (± 3.5)	785	550
"	10.0	2686 (± 58)	100 (± 2.8)	500	500
Caffeic acid	0.1	5762 (± 62)	212 (± 6.3)	848	400
"	1.0	5478 (± 51)	198 (± 5.5)	1287	650
"	10.0	4766 (± 47)	180 (± 4.5)	585	325
Ferulic acid	0.1	6527 (± 36)	194 (± 4.3)	776	400
"	1.0	5258 (± 38)	178 (± 4.8)	1112	625
"	10.0	4802 (± 50)	165 (± 7.0)	495	300
Cinnamic acid	0.1	5195 (± 41)	201 (± 6.1)	1105	550
"	1.0	3920 (± 28)	137 (± 5.4)	685	500
"	10.0	987 (± 18)	43 (± 2.7)	183	425
p-Coumaric acid	0.1	4148 (± 43)	138 (± 3.2)	690	500
"	1.0	3643 (± 20)	124 (± 4.1)	744	600
"	10.0	3166 (± 18)	121 (± 5.3)	514	425

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

Figs. C-14 to C-19 and Table 18 represent effect of phenolic acids in absence of 2,4-D on growth and polyphenol production in callus cultures of Crotalaria. p-Hydroxybenzoic acid and chlorogenic acid at low concentrations tested (0.1 and 1.0 mg/l) stimulated growth over the control (MS medium without 2,4-D - supported 925 mg fresh weight and 53 mg dry weight) as determined by fresh and dry weights. On the other hand, low levels of caffeic and p-coumaric acids had marginal effect on growth, while ferulic acid and cinnamic acid were found inhibitory. Whereas with increasing levels of other phenolic acids more pronounced inhibition of growth was observed, increasing levels of caffeic acid stimulated growth. The production of phenolic compounds per culture was suppressed at higher concentration (10.0 mg/l) by all the phenolic acids tested except caffeic acid. On unit basis, p-hydroxybenzoic, caffeic, ferulic and p-coumaric acids enhanced phenolic accumulation with increasing concentrations, while chlorogenic and cinnamic acids supported more polyphenols at 1.0 mg/l than 10.0 mg/l. Tissues grown on p-hydroxybenzoic, caffeic, ferulic and p-coumaric acids accumulated (0.925, 1.05, 1.0 and 0.975% respectively) more polyphenols as

Fig. C-14 to Fig. C-19. Effect of different phenolic acids at 0.1, 1.0 and 10.0 mg/l concentrations in absence of 2,4-D on growth and polyphenol production in Crotalaria callus cultures.

Inoculum : 300± 30 mg tissue by fresh weight on 30 ml MS medium.

Other experimental details as given in Table 18.

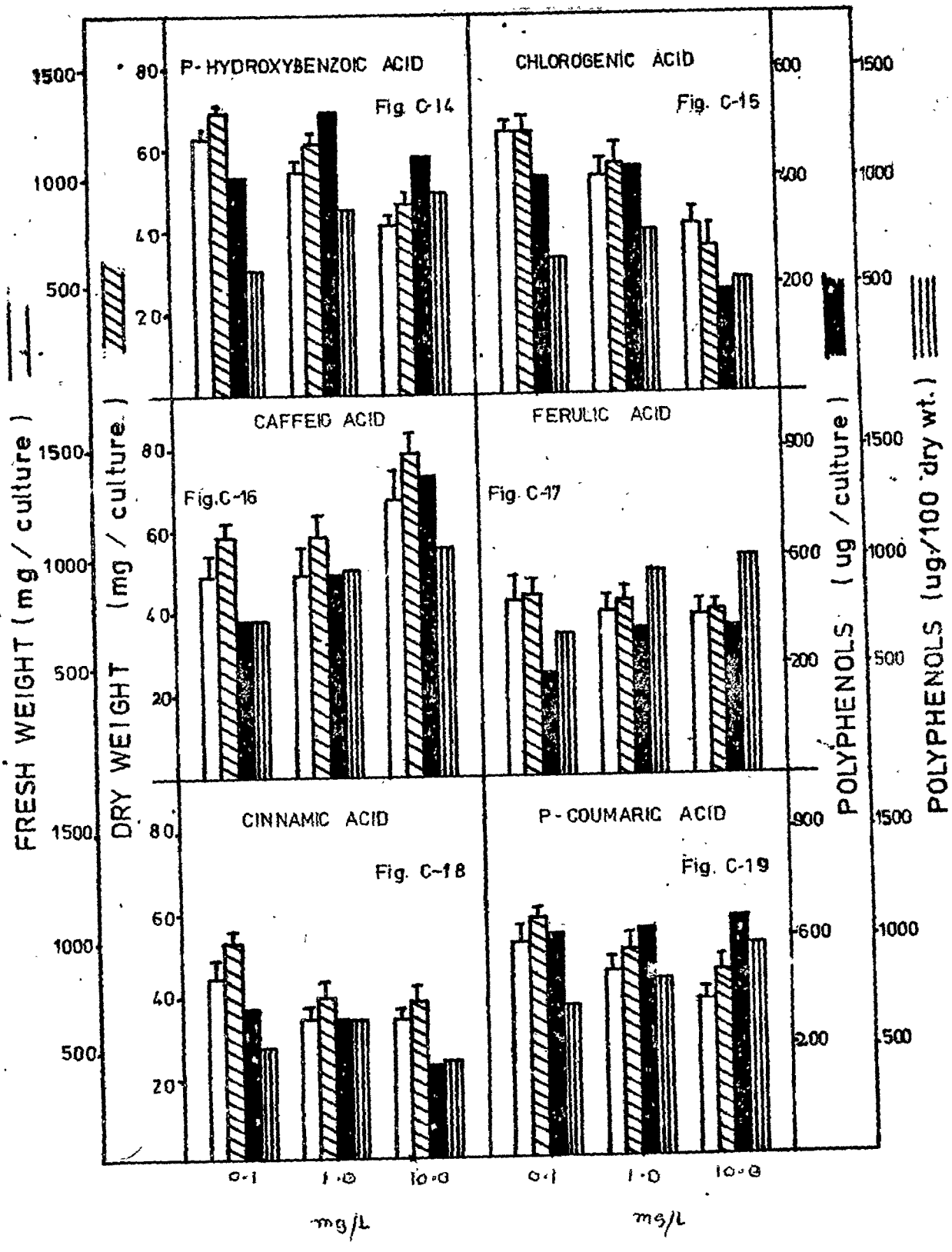

P-HYDROXYBENZOIC ACID
Fig. C-14
CHLOROGENIC ACID
Fig. C-15
CAFFEIC ACID
Fig. C-16
FERULIC ACID
Fig. C-17
CINNAMIC ACID
Fig. C-18
P-COUMARIC ACID
Fig. C-19
FRESH WEIGHT (mg / culture)
DRY WEIGHT (mg / culture)
POLYPHENOLS (ug / culture)
POLYPHENOLS (ug/100 dry wt.)
0·1
1·0
10·0
mg/L

Table 18 : Effect of Phenolic Acids in Absence of 2,4-D on Growth and Polyphenol Production in Crotalaria Callus Cultures *

Inoculum : 300± 30 mg tissue by fresh weight on 30 ml MS medium supplemented with 2.0% sucrose and phenolic acids as given below :

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

Phenolic acid	Concentration (mg/l)	Fresh Wt. (mg)	Dry Wt. (mg)	Polyphenols	
				ug/culture	ug/100 mg dry wt.
p-Hydroxybenzoic acid	0.1	1175 (± 22)	69 (± 1.2)	396	575
"	1.0	1030 (± 20)	61 (± 1.8)	518	850
"	10.0	773 (± 18)	47 (± 2.2)	435	925
Chlorogenic acid	0.1	1198 (± 19)	64 (± 3.1)	400	625
"	1.0	1006 (± 28)	56 (± 4.0)	420	750
"	10.0	776 (± 30)	36 (± 3.8)	189	525
Caffeic acid	0.1	925 (± 32)	59 (± 2.6)	428	725
"	1.0	927 (± 48)	58 (± 3.5)	551	950
"	10.0	1262 (± 52)	79 (± 4.2)	829	1050
Ferulic acid	0.1	797 (± 43)	44 (± 3.0)	286	650
"	1.0	754 (± 27)	42 (± 2.5)	399	950
"	10.0	731 (± 31)	40 (± 1.8)	400	1000
Cinnamic acid	0.1	835 (± 33)	53 (± 2.2)	278	525
"	1.0	656 (± 16)	40 (± 3.0)	260	650
"	10.0	641 (± 18)	39 (± 2.8)	175	450
p-Coumaric acid	0.1	987 (± 32)	59 (± 1.9)	413	700
"	1.0	852 (± 25)	51 (± 3.1)	421	825
"	10.0	721 (± 15)	45 (± 2.8)	439	975

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

compared to control medium (MS medium without 2,4-D - supported 0.9% polyphenols).

SUMMARY

It is clear from the results presented in this section that of the different sugars tested as energy source for growth and polyphenol production sucrose was the most efficient. Among the inorganic nitrogen sources examined, ammonium salt inhibited growth and potassium salt inhibited polyphenol production. However, a balanced supply of ammonium and potassium nitrates was more effective than other sources both for the growth of the cells and also for the polyphenol production. Among the organic nitrogen sources tested, yeast extract at 5 g/l concentration supported maximum growth and polyphenol production. Growth and polyphenol production supported by yeast extract as a sole nitrogen source were higher than that by best inorganic nitrogen source (i.e. a balanced supply of ammonium and potassium nitrates). However, inorganic nitrogen source was preferred over yeast extract for further experiments because of its chemically defined constitution. Urea at

all the concentrations tested failed to enhance growth and phenolic production.

L-phenylalanine and L-tyrosine, the precursors in the biosynthesis of phenylpropanoid compounds, were tested for their ability to support growth and polyphenol production. It was observed that both the amino acids failed to enhance growth over the control. Though capacity of cells to accumulate polyphenols increased with L-phenylalanine medium, overall production of phenolic compounds was less than the control. On the other hand, L-tyrosine at 0.1% concentration promoted maximum polyphenol synthesis among all the nutritional factors examined so far for their ability to support polyphenol production. With L-tyrosine (0.1%) medium, the accumulation capacity of the cells (3.6%) for phenolic compounds was also maximum.

Examination of the effect of six phenolic acids on polyphenol production and growth revealed that phenolic acids were inhibitory to growth and polyphenol production in presence of 2,4-D. However, in absence of 2,4-D only ferulic and cinnamic acids were effective growth inhibitors at low concentrations. Caffeic acid enhanced maximum polyphenol production, both per culture as well as on unit basis, over the control at the highest level tested.

D. PROGRESSIVE CHANGES IN GROWTH, ACCUMULATION OF PHENOLIC MATERIALS AND THE DEVELOPMENT OF RELATED ENZYME ACTIVITIES UNDER DIFFERENT CULTURAL CONDITIONS

It was well established in previous two sections that Crotalaria callus cultures can synthesize and accumulate phenolic compounds during their growth in culture. The investigation was further extended to study the progressive changes in growth and phenolic compounds.

Many tissues respond in a consistent way to a variety of environmental stimuli by modifying the activity of certain enzymes. In this section progressive changes in the enzymes related to phenylpropanoid compounds such as PAL, TAL, p-coumaryl CoA : ligase and transaminases, peroxidase and IAA oxidase, which are also known for their role in the physiology of growth, are examined during the course of culture period. The relationship of their changing patterns with the synthesis of phenolic materials under various cultural conditions are discussed.

Influence of different concentrations of sucrose, 2,4-D, NAA, GA_3, kinetin and cyclehexinude are studied on growth, phenolic accumulation and the development of above mentioned enzymes. Further, the experiments designed to

study the hormonal effect in presence and absence of light are also included in this section.

1. Effect of Sucrose Concentrations on Growth, Polyphenol Synthesis and the Development of Peroxidase, IAA Oxidase, PAL and TAL Enzyme Activities in Crotalaria Callus Cultures.

Callus pieces weighing 300± 30 mg by fresh weight were separately inoculated onto 30 ml MS medium (Table 1, Chapter II) supplemented with 0.5, 2 or 4 per cent sucrose in addition to 2.0 mg/l 2,4-D.

After incubation for 30 days in light at a constant temperature of 26± 2°C, a fixed number of replicate culture vessels was harvested at 5 days interval upto day 30. The tissues of each treatment were pooled separately for the measurement of growth and total phenolic compounds, and for assaying peroxidase, IAA oxidase, PAL and TAL activities as described in Chapter II, Materials and Methods, 5A, 5B, 6A, 7A, 7B and 7C.

The growth response and the synthesis of phenolic materials in the tissues as influenced by different sucrose levels is shown in Figs. D-1 to D-4 and Table 19. Growth

and polyphenol accumulation was low during the early culture period. While increase in sucrose content of the medium led to increase in polyphenol synthesis, 2.0% sucrose supported maximum growth as measured by fresh and dry weights. The most rapid growth was observed between 10th - 20th day period on 2 and 4 % sucrose media, whereas it was between 5 and 15th day on 0.5% sucrose medium. The maximum polyphenol production was recorded during the period of rapid growth (10 to 20 days in case of 2 and 4 % sucrose and 5 to 15 days in case of 0.5% sucrose). In case of low sucrose medium polyphenol synthesis was registered in the early part of culture period after which it slowly declined. Clearly, polyphenol production was dependent on the availability of carbohydrate and depletion in sucrose led to decrease in the synthesis of phenolic materials.

The data replotted on percentage basis, as illustrated in Fig. D-4, showed that in 0.5 and 2% sucrose media polyphenol synthesis terminated after day 15; while the synthesis continued till day 30 in 4% sucrose medium. Maximum 1.0, 1.1 and 1.45% synthesis of phenolic compounds were recorded in 0.5, 2 and 4% sucrose media respectively.

Table 19 : Progressive Changes in Growth and Polyphenol Production at Different Sucrose Concentrations*.

Inoculum : 300± 30 mg tissue by fresh weight on 30 ml MS medium supplemented with 0.5, 2.0 and 4.0 % of sucrose in addition to 2.0 mg/l 2,4-D.

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

Time (day)	0.5%				2.0%				4.0%			
	Fresh wt. (mg)	Dry wt. (mg)	Polyphenols ug/cult.	Polyphenols ug/100 mg dry wt.	Fresh wt. (mg)	Dry wt. (mg)	Polyphenols ug/cult.	Polyphenols ug/100 mg dry wt.	Fresh wt. (mg)	Dry wt. (mg)	Polyphenols ug/cult.	Polyphenols ug/100 mg dry wt.
0	300 (±30)	11.00 (±2.8)	88	800	300 (±30)	11.00 (±2.8)	88	800	300 (±30)	11.00 (±2.8)	88	800
5	445 (±29)	16.11 (±4.2)	113	700	412 (±32)	17.00 (±3.2)	144	850	436 (±31)	15.90 (±3.8)	143	900
10	978 (±71)	36.16 (±3.9)	289	800	1300 (±81)	51.15 (±2.6)	473	925	1140 (±56)	46.13 (±4.7)	507	1100
15	1876 (±52)	68.05 (±5.4)	680	1000	3845 (±76)	135.00 (±4.2)	1485	1100	3562 (±48)	136.31 (±5.3)	1704	1250
20	2235 (±38)	81.00 (±6.1)	648	800	5876 (±58)	189.53 (±5.1)	1848	975	5383 (±57)	197.84 (±6.8)	2572	1300
30	2725 (±76)	101.16 (±6.9)	556	550	6772 (±58)	229.00 (±7.8)	1889	825	6217 (±63)	225.96 (±8.1)	3276	1450

* Data represent average of six replicates.
Figures in the paranthesis represent standard errors.

Fig. D-1 to D-6. Changes in growth as measured by fresh (Fig. D-1) and dry (Fig. D-2) weights, accumulation of total (Fig. D-3) and mean (Fig. D-4) phenolic compounds and development of total (Fig. D-5) and specific (Fig. D-6) activity in Crotalaria callus cultures at different sucrose concentrations.

Treatments : MS medium with 0.5, 2.0 or 4.0 % sucrose in addition to 2.0 mg/l 2,4-D.

-□- 0.5% sucrose,

-○- 2.0% sucrose, and

-△- 4.0% sucrose.

Experimental details as given in Table 19 and Table 20.

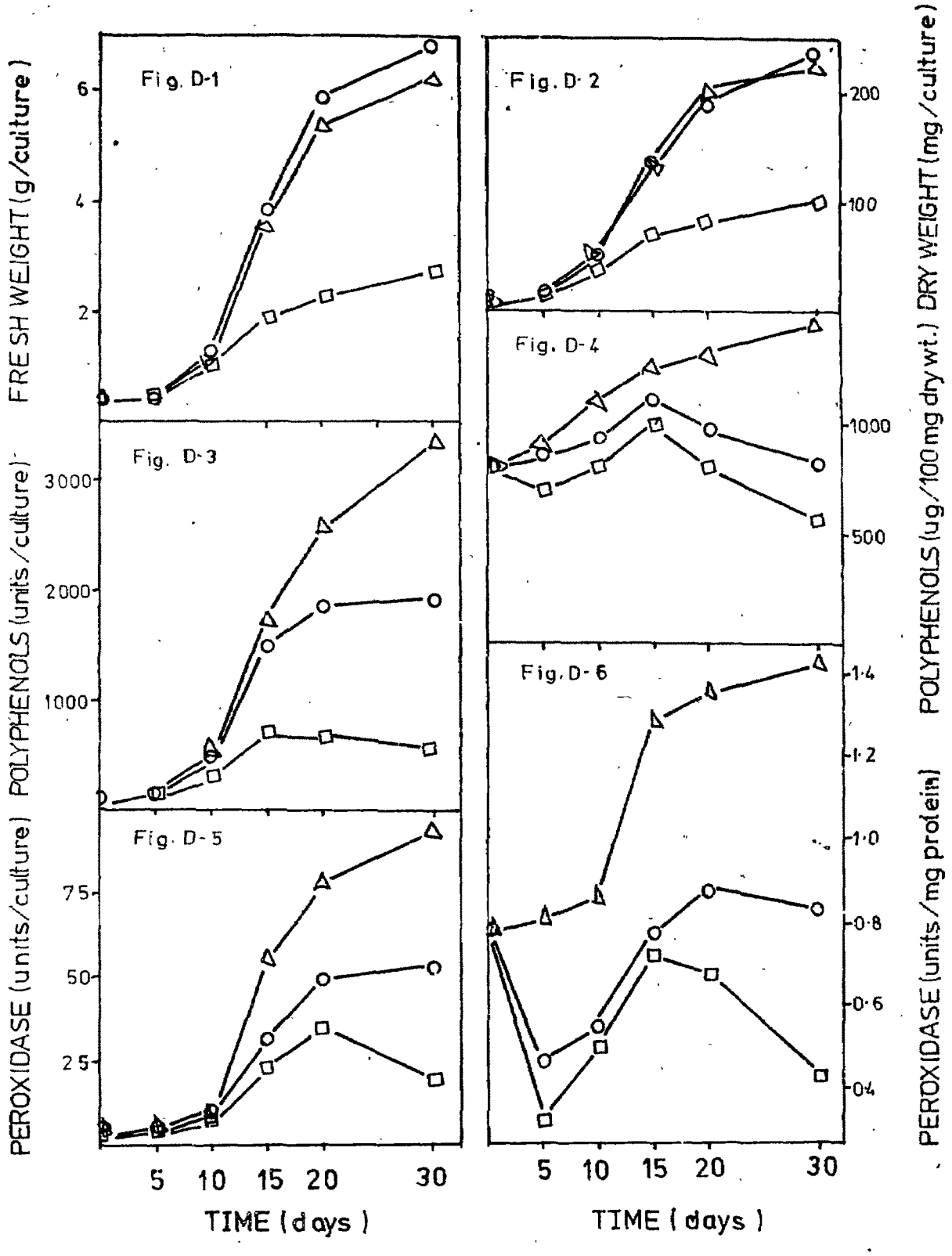


Fig. D-1
Fig. D-2
Fig. D-3
Fig. D-4
Fig. D-5
Fig. D-6
FRESH WEIGHT (g/culture)
DRY WEIGHT (mg/culture)
POLYPHENOLS (units/culture)
POLYPHENOLS (ug/100 mg dry wt.)
PEROXIDASE (units/culture)
PEROXIDASE (units/mg protein)
TIME (days)
TIME (days)

When changes in peroxidase activity were studied at different sucrose concentrations, it was observed that the increase in the sucrose level also enhanced peroxidase activity (Fig. D-5 and Table 20). Maximum increase in peroxidase activity was recorded from day 10 to 20. Progressive changes in peroxidase activity showed a close correlation with the accumulation of total phenolics in the tissues particularly at 2 and 4% sucrose levels. This correlation suggested an important role of the peroxidase in phenolic biosynthesis.

In case of low and high sucrose concentrations, specific activity of peroxidase (Fig. D-6) quite closely correlated with the accumulation of phenolic compounds in the tissues on unit basis. At 2% sucrose level an initial suppression of enzyme activity was registered in contrast to the polyphenol synthesis where no suppression was recorded during this early culture period. Further, though polyphenol accumulation terminated on day 15, peroxidase continued to rise till day 20 at this sucrose level. However, during the remaining part of the culture period similar patterns were observed in both the cases.

Unlike peroxidase, the IAA oxidase activity

Table 20 : Changes in Peroxidase and IAA Oxidase Activities at Different Levels of Sucrose.

Inoculum : 300± 30 mg tissue by fresh weight on 30 ml MS medium supplemented with 0.5, 2.0 and 4.0 % sucrose in addition to 2.0 mg/l 2,4-D.

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

Time (day)	0.5%				2.0%				4.0%			
	Peroxidase		IAA Oxidase		Peroxidase		IAA Oxidase		Peroxidase		IAA Oxidase	
	units/ cult.	units/ mg prot.	units/ cult.	units/ mg prot.	units/ cult.	units/ mg prot.	units/ cult.	units/ mg prot.	units/ cult.	units/ mg prot.	units/ cult.	units/ mg prot.
0	2.26	0.79	1.66	0.58	2.26	0.79	1.66	0.58	2.26	0.79	1.66	0.58
5	2.56	0.32	6.73	0.84	3.96	0.47	8.05	0.96	5.12	0.81	11.54	1.82
10	7.32	0.49	17.02	1.15	9.75	0.54	20.07	1.34	11.53	0.85	25.03	1.86
15	23.22	0.72	50.50	1.58	31.15	0.77	69.13	2.15	55.15	1.28	95.98	2.22
20	35.55	0.66	22.98	0.43	50.53	0.88	41.79	0.72	78.75	1.34	74.10	1.27
30	20.11	0.43	9.39	0.20	52.82	0.83	35.65	0.56	92.22	1.42	52.53	0.81

One Unit of Peroxidase Activity = change in OD of 10/min.

One Unit of IAA Oxidase Activity = 1 mg of IAA destroyed/25 min at 37°C.

increased from the beginning (Fig. D-7 and Table 20). It continued to rise in all the treatments attaining peak activity on day 15. Increase in IAA oxidase activity during 5 to 15 days period corresponded with the accumulation of polyphenols. The activity declined after day 15. As in the case of peroxidase, the maximum IAA oxidase activity was registered in higher sucrose level (4 %) which also supported maximum polyphenol synthesis. The developmental pattern of specific activity of IAA oxidase at all the sucrose levels (Fig. D-8) was very similar to that of total IAA oxidase activity per culture. 5 to 15 day period of enhanced IAA oxidase activity corresponded with polyphenol production during that period.

The effect of sucrose concentration on the development of PAL and TAL activities are presented in Table 21 and illustrated in Figs. D-9 to D-12. As compared to PAL activity, TAL activity was low throughout the culture period. Both PAL and TAL activities were low during the early stages of growth (upto 10 days) at all the sucrose concentrations. However, the subsequent development corresponded quite closely with the rate of polyphenol synthesis upto day 15. Further, though in the case of

Table 21 : Influence of Different Sucrose Levels on Progressive Changes in PAL and TAL Activities.

Inoculum : 300± 30 mg tissue by fresh weight on 30 ml MS medium supplemented with 0.5, 2.0 and 4.0 % sucrose in addition to 2.0 mg/l 2,4-D.

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

Time (day)	0.5%				2.0%				4.0%			
	PAL		TAL		PAL		TAL		PAL		TAL	
	units/ cult.	units/ mg prot.	units/ cult.	units/ mg prot.	units/ cult.	units/ mg prot.	units/ cult.	units/ mg prot.	units/ cult.	units/ mg prot.	units/ cult.	units/ mg prot.
0	0.27	0.095	0.067	0.024	0.27	0.095	0.067	0.024	0.27	0.095	0.067	0.024
5	0.84	0.105	0.230	0.029	0.93	0.111	0.245	0.029	1.01	0.160	0.253	0.040
10	1.87	0.126	0.482	0.032	2.76	0.152	0.707	0.038	3.46	0.256	0.858	0.063
15	12.90	0.403	3.685	0.115	27.45	0.680	7.625	0.189	48.25	1.123	12.697	0.295
20	8.69	0.163	2.780	0.052	17.19	0.298	5.056	0.087	30.76	0.526	8.544	0.146
30	3.86	0.082	1.076	0.023	6.27	0.098	1.590	0.025	29.06	0.450	8.303	0.128

One Unit of PAL Activity = formation of 10 umoles of cinnamate/hr at 37°C.

One Unit of TAL Activity = formation of 10 umoles of p-coumarate/hr at 37°C.

Fig. D-7 to D-12. The development of total (Fig. D-7) and specific (Fig. D-8) activities of IAA oxidase, PAL (Fig. D-9 and Fig. D-10) and TAL (Fig. D-11 and (Fig. D-12) as influenced by various sucrose concentrations.

Treatments : MS medium supplemented with 0.5, 2.0 or 4.0 % sucrose in addition to 2.0 mg/l 2,4-D.

-□- 0.5% sucrose,

- ○ -2.0% sucrose, and

-△- 4.0% sucrose.

Other experimental details as given in Table 20 and Table 21.

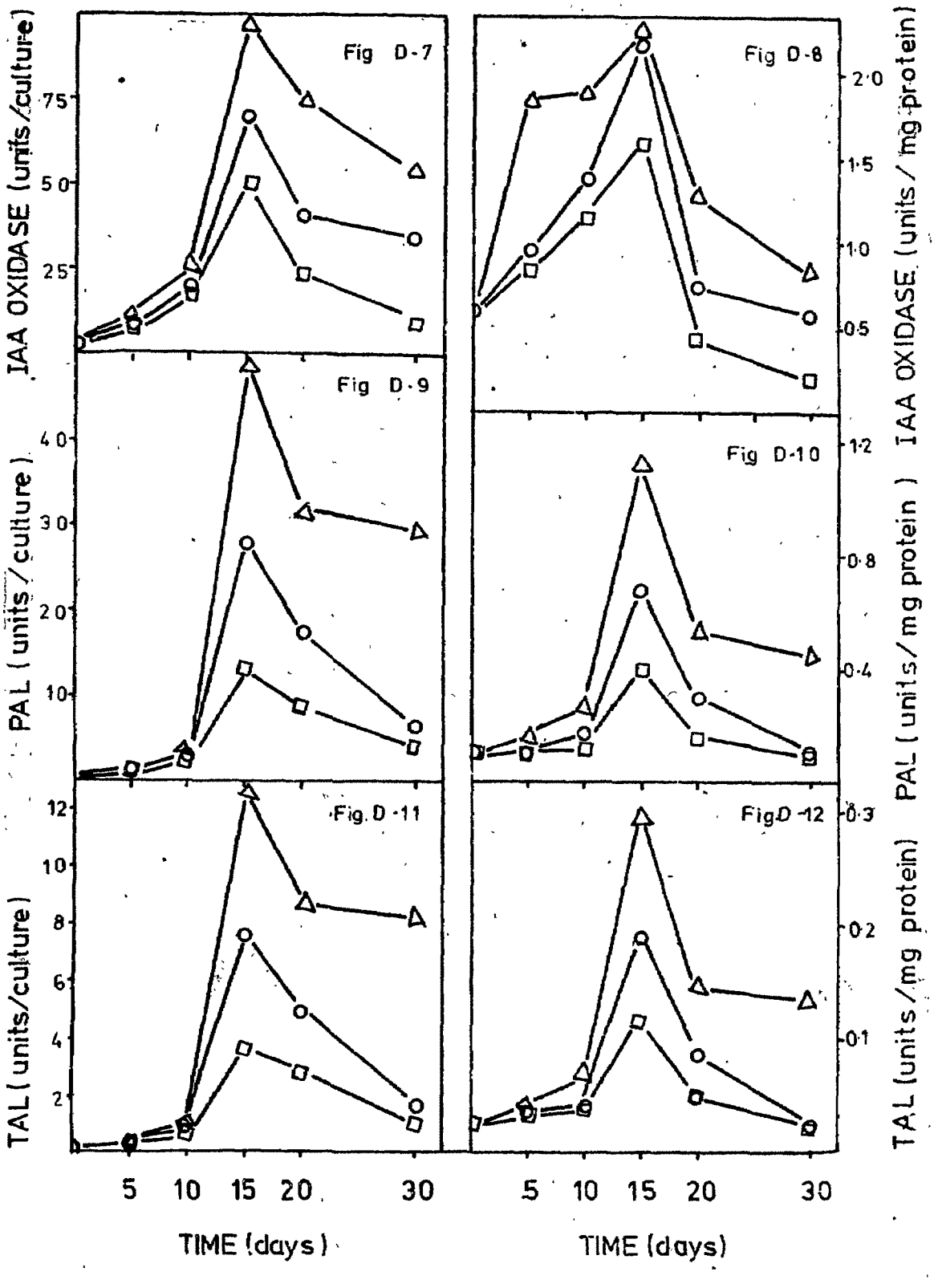


Fig D-7
Fig D-8
Fig D-9
Fig D-10
Fig. D-11
Fig D-12
IAA OXIDASE (units/culture)
PAL (units/culture)
TAL (units/culture)
IAA OXIDASE (units/mg protein)
PAL (units/mg protein)
TAL (units/mg protein)
TIME (days)
TIME (days)

2 and 4 % sucrose levels polyphenols continued to increase after day 15, PAL and TAL showed a sharp decline in the activity. Close correlation was observed only at low sucrose concentration where PAL, TAL and polyphenols terminated on day 15.

Evaluation of the data on relative amount of polyphenols (Fig. D-4) and specific activities of PAL (Fig. D-10) and TAL (Fig. D-12) indicated clear correlation between them at 0.5 and 2 % sucrose levels. However, at 4 % sucrose, while PAL and TAL activities declined sharply after 15 day, polyphenol synthesis continued to rise.

2. <u>2,4-D Concentration Effect on Growth, Polyphenol Production and the Development of Peroxidase, IAA Oxidase, PAL and TAL Activities in Presence and Absence of light.</u>

MS medium (Table 1, Chapter II) was supplemented with 0.2, 2.0 and 10.0 mg/l 2,4-D in addition to 2 % sucrose to study the effect of 2,4-D level on the production of polyphenols and the development of the enzymes. Weighed amount of callus pieces were inoculated into 150 ml culture flasks on 30 ml MS medium supplemented

Table 22 : Effect of Light and Dark at Three 2,4-D Concentrations on Growth of *Crotalaria* Callus Cultures*
Inoculum : 300± 30 mg tissue by fresh weight on 30 ml MS medium supplemented with 0.2, 2.0 and 10.0 mg/l 2,4-D.
Incubation : 30 days at 26± 2°C.

Time (day)	2,4-D Conc. (mg/l)	Growth			
		In Light (1000 Lux)		In Dark	
		Fresh wt. (mg)	Dry wt. (mg)	Fresh wt. (mg)	Dry wt. (mg)
0	-	300 (±30)	11.00 (±2.8)	300 (±30)	11.00 (±2.8)
5	0.2	402 (±41)	16.08 (±4.8)	412 (±43)	16.15 (±3.6)
	2.0	412 (±32)	17.00 (±3.2)	415 (±41)	17.18 (±3.9)
	10.0	410 (±28)	16.52 (±3.6)	421 (±48)	19.50 (±3.2)
10	0.2	1009 (±71)	40.15 (±4.3)	1125 (±58)	43.45 (±5.2)
	2.0	1300 (±81)	51.15 (±2.6)	1421 (±53)	58.00 (±4.4)
	10.0	808 (±33)	35.12 (±4.2)	875 (±62)	38.12 (±4.5)
15	0.2	2015 (±62)	72.31 (±5.8)	2126 (±57)	74.00 (±3.9)
	2.0	3845 (±76)	135.00 (±4.2)	3965 (±72)	141.26 (±6.3)
	10.0	1541 (±46)	62.23 (±5.1)	1736 (±51)	71.16 (±5.7)
20	0.2	3616 (±57)	119.96 (±3.8)	3836 (±56)	137.00 (±6.8)
	2.0	5876 (±58)	189.53 (±5.1)	5987 (±86)	203.00 (±6.2)
	10.0	2125 (±53)	73.00 (±6.9)	2450 (±49)	84.00 (±7.2)
30	0.2	4625 (±38)	156.00 (±6.8)	4975 (±81)	180.11 (±7.8)
	2.0	6772 (±58)	229.00 (±7.8)	7015 (±98)	242.15 (±8.9)
	10.0	2689 (±68)	86. 00 (±7.9)	3132 (±73)	109.25 (±6.8)

* Data represent average of six replicates.
Figures in the paranthesis represent standard errors.

Fig. D- 13. Effect of different 2, 4-D concentrations on growth (fresh and dry weights) in presence and absence of light.

Treatments : MS medium with 0.2, 2.0 or 10.0 mg/l 2,4-D.

-□- 0.2 mg/l 2,4-D - light,

-■- 0.2 " " - dark,

-O- 2.0 " " - light,

-●- 2.0 " " - dark,

-Δ- 10.0 " " - light, and

-▲- 10.0 " " - dark.

Experimental details as given in Table 22.

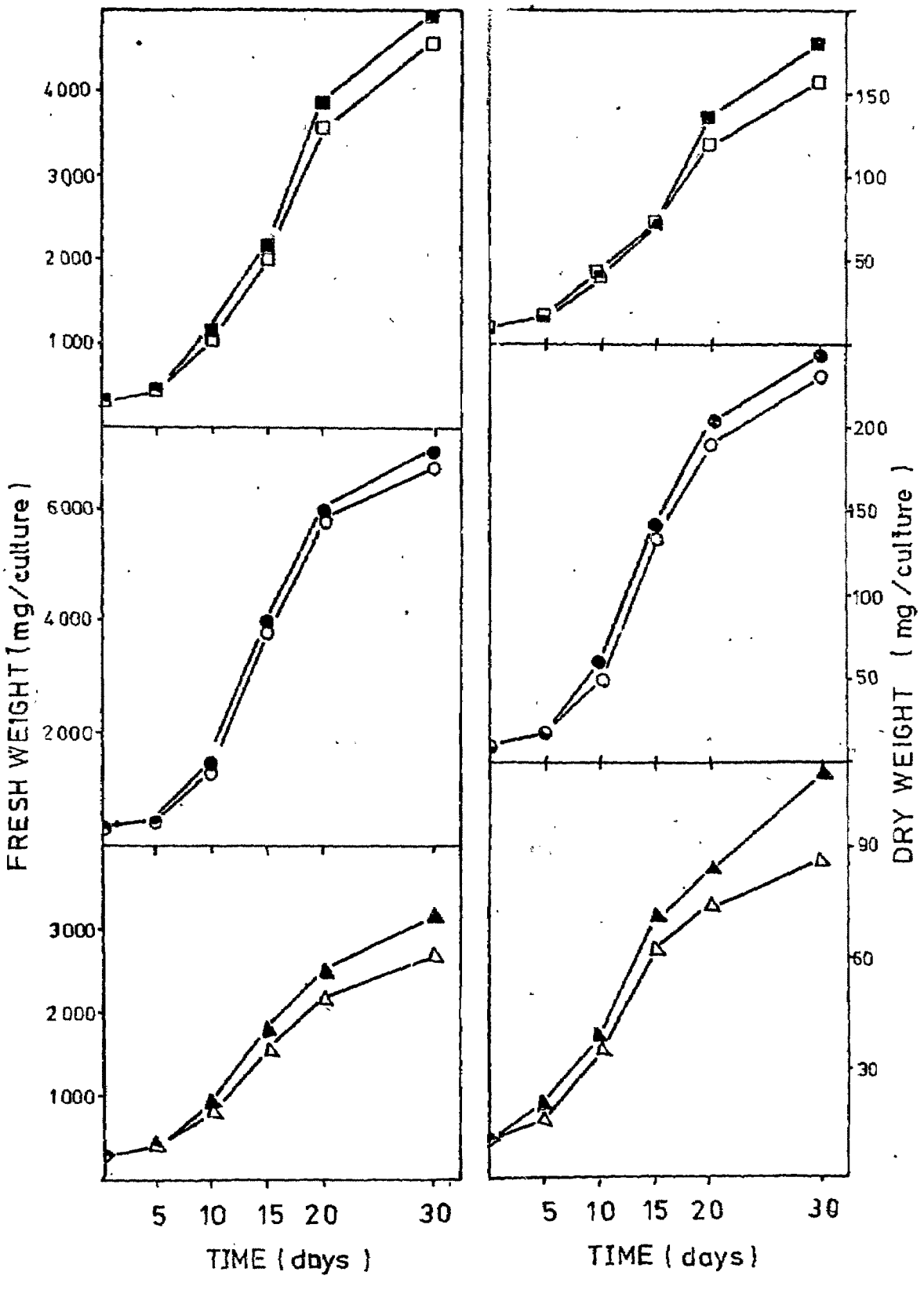

FRESH WEIGHT (mg/culture)
DRY WEIGHT (mg/culture)
4000
3000
2000
1000
6000
4000
2000
3000
2000
1000
150
100
50
200
150
100
50
90
60
30
5
10
15
20
30
TIME (days)
TIME (days)

Fig. D-13

as above. The flasks were incubated under 1000 Lux light given from cool white flourescent lamps. An another set of replicates was incubated in complete darkness. In both the cases the temperature ($26 \pm 2^{o}C$) and other conditions were uniformly maintained.

A fixed number of replicate flasks was harvested at 5 days intervals upto 30 days for the measurement of growth, estimation of total phenolic compounds and assaying peroxidase, IAA oxidase, PAL and TAL activities.

More growth of the tissues was recorded in the dark than in light at all 2,4-D concentrations (Fig. D-13 and Table 22). 2.0 mg/l 2,4-D level supported maximum growth in light as well as in dark. Growth attained on 10.0 mg/l 2,4-D was less than that attained on 0.2 mg/l 2,4-D levels, except during initial 5 days. Suppression of growth at high auxin level was more pronounced in light.

In general, light enhanced polyphenol synthesis at all the 2,4-D levels tested (Fig. D-14 and Table 23). Polyphenol synthesis was considerably reduced at high 2,4-D (10.0 mg/l) concentration. The highest synthesis of phenolic compounds was registered in tissues growth

Table 23 : Effect of 2,4-D on Polyphenol Production in Presence and Absence of Light.
Inoculum : 300± 30 mg tissue by fresh weight on 30 ml MS medium supplemented with 0.2, 2.0 and 10.0 mg/l 2,4-D.
Incubation : 30 days at 26± 2°C.

Time (days)	2,4-D Conc. (mg/l)	Polyphenols			
		In Light (1000 Lux)		In Dark	
		ug/culture	ug/100 mg dry wt.	ug/culture	ug/100 mg dry wt.
0	-	88	800	88	800
5	0.2	133	825	121	750
	2.0	140	825	133	775
	10.0	120	725	132	675
10	0.2	341	850	337	775
	2.0	473	925	464	800
	10.0	263	750	219	575
15	0.2	651	900	592	800
	2.0	1485	1100	1201	850
	10.0	545	875	374	525
20	0.2	960	800	890	650
	2.0	1848	975	1624	800
	10.0	565	775	399	475
30	0.2	1014	650	946	525
	2.0	1889	825	1756	725
	10.0	580	675	492	450

Fig. D-14. Accumulation of total and relative amount of phenolic materials at various 2,4-D concentrations in presence and absence of light.

Treatments : MS medium with 0.2, 2.0 or 10.0 mg/l 2,4-D.

- □ - 0.2 mg/l 2,4-D - light,
- ■ - 0.2 " " - dark,
- ○ - 2.0 " " - light,
- ● - 2.0 " " - dark,
- △ - 10.0 " " - light, and
- ▲ - 10.0 " " - dark.

Experimental details as given in Table 23.

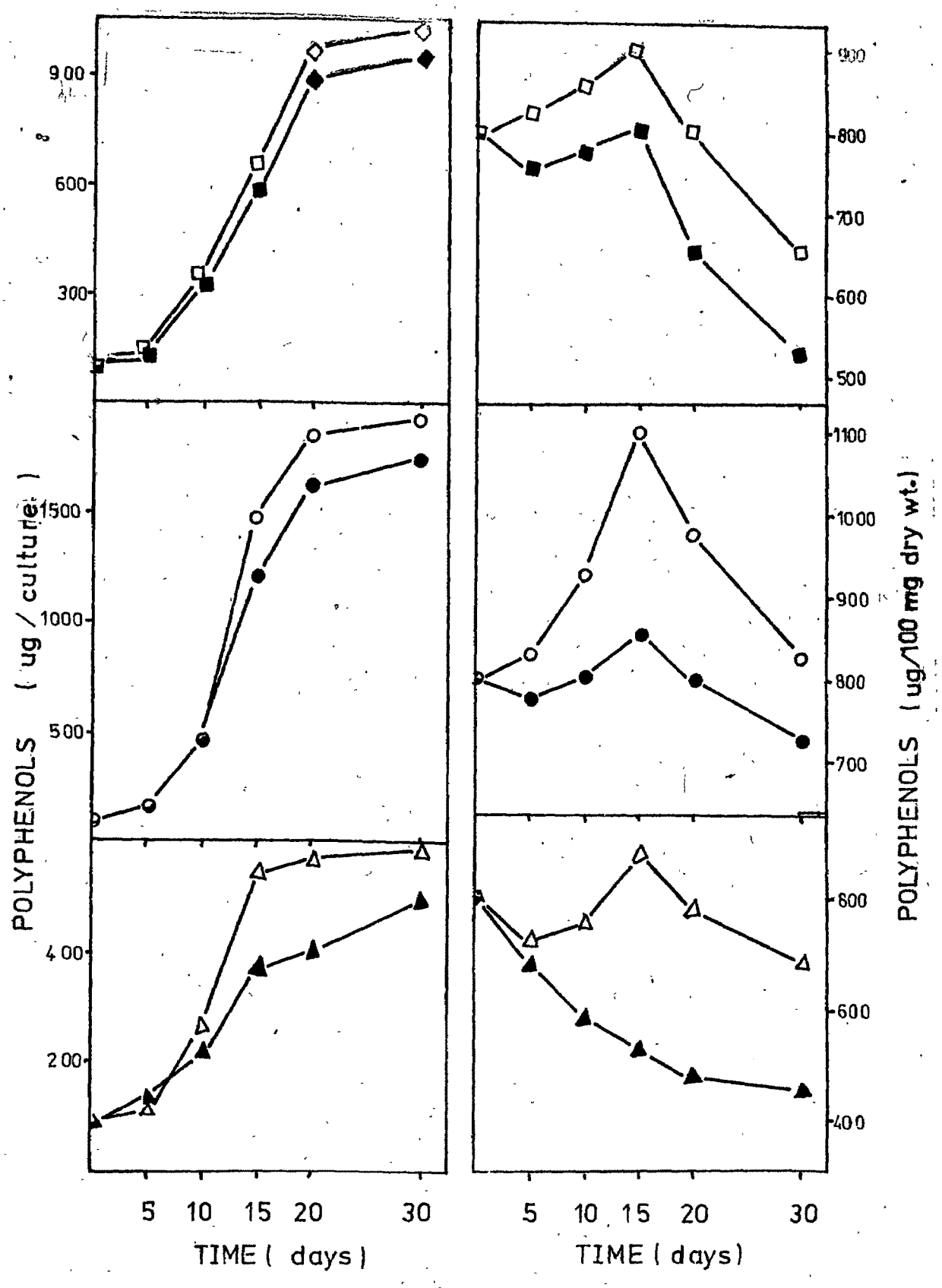

POLYPHENOLS (ug / culture)
POLYPHENOLS (ug/100 mg dry wt.)
TIME (days)
TIME (days)
5
10
15
20
30
900
600
300
1500
1000
500
400
200
800
700
1100
900
600
400

Fig. D-14

in light on 2.0 mg/l 2,4-D medium. The promotory effect of light on polyphenol production was more pronounced and marked at higher 2,4-D level than at lower auxin level. Thus, in 0.2 mg/l 2,4-D medium 6.7% stimulation was observed while 15.17% stimulation was recorded at 10.0 mg/l 2,4-D level in light over dark after the culture period of 30 days.

On percentage basis, highest polyphenol accumulation was registered at 2.0 mg/l 2,4-D level on day 15 (1.1%) (Fig. D-14 and Table 23) in light. On the other hand, this concentration supported only 0.85% polyphenol synthesis in dark. Inhibitory effect of dark was more marked at higher 2,4-D level.

Fig. D-15 and Table 24 illustrate the effect of light and dark at different 2,4-D concentrations on peroxidase activity. The higher peroxidase activity was recorded in the tissues with greater accumulation of polyphenols. In case of 0.2 and 2.0 mg/l 2,4-D concentrations, enhanced enzyme activity was observed in light. However, at higher 2,4-D concentration the level of enzyme activity recorded in light and in dark grown

Table 24 : Effect of Light and Dark on Peroxidase Activity in *Crotalaria* Callus Cultures Grown on Three 2,4-D Concentrations.

Inoculum : 300± 30 mg tissue by fresh weight on 30ml MS medium supplemented with 0.2, 2.0 and 10.0 mg/l 2,4-D.

Incubation : 30 days at 26± 2°C.

Time (day)	2,4-D Conc. (mg/l)	Peroxidase *			
		In Light (1000 Lux)		In Dark	
		units/cult.	units/mg prot.	units/cult.	units/mg prot.
0	-	2.26	0.79	2.26	0.79
5	0.2	3.08	0.45	2.19	0.67
	2.0	3.96	0.47	2.52	0.84
	10.0	2.46	0.42	2.58	0.87
10	0.2	6.21	0.48	5.25	0.58
	2.0	9.75	0.54	7.38	0.93
	10.0	5.03	0.46	7.06	0.92
15	0.2	20.00	0.62	15.15	0.53
	2.0	31.15	0.77	28.13	1.06
	10.0	17.00	0.58	16.95	0.66
20	0.2	24.12	0.52	22.36	0.51
	2.0	50.53	0.88	39.26	0.97
	10.0	17.85	0.43	18.00	0.45
30	0.2	20.11	0.38	19.18	0.32
	2.0	52.82	0.83	33.06	0.67
	10.0	18.82	0.39	19.12	0.41

* One Unit of Peroxidase Activity = change in OD of 10/min.

tissues was nearly equal. Maximum increase in peroxidase activity in light as well as in dark at all the 2,4-D levels was registered during the period of rapid growth (10 - 20 days) which also corresponded with the period of maximum phenolic production.

On specific activity basis (Fig. D-15, Table 24), suppression in peroxidase activity was recorded in light for initial 5 days after which it enhanced again giving a peak on day 15 at 0.2 and 10.0 mg/l and on day 20 at 2.0 mg/l 2,4-D. On the other hand, in dark at low 2,4-D level a continuous fall in specific activity was recorded. However, at optimal and high 2,4-D levels in dark, initial increase was registered followed by a sharp decrease after day 15 in tissues grown on 2.0 mg/l and after day 10 on 10.0 mg/l 2,4-D level.

Influence of 2,4-D concentrations on IAA oxidase activity is illustrated in Fig. D-16 and presented in Table 25. Unlike peroxidase, there was sharp increase in IAA oxidase activity upto day 15 at all 2,4-D levels tested both in light and in dark. As was the case with peroxidase activity, IAA oxidase activity stimulated by

Fig. D-15. Effect of light and dark on the development of peroxidase and its specific activity at various 2,4-D levels in Crotalaria callus cultures.

Treatments : MS medium supplemented with 0.2, 2.0 or 10.0 mg/l 2,4-D.

- □ - 0.2 mg/l 2,4-D - light,
- ■ - 0.2 " " - dark,
- ○ - 2.0 " " - light,
- ● - 2.0 " " - dark,
- △ - 10.0 " " - light, and
- ▲ - 10.0 " " - dark.

Experimental details as given in Table 24.

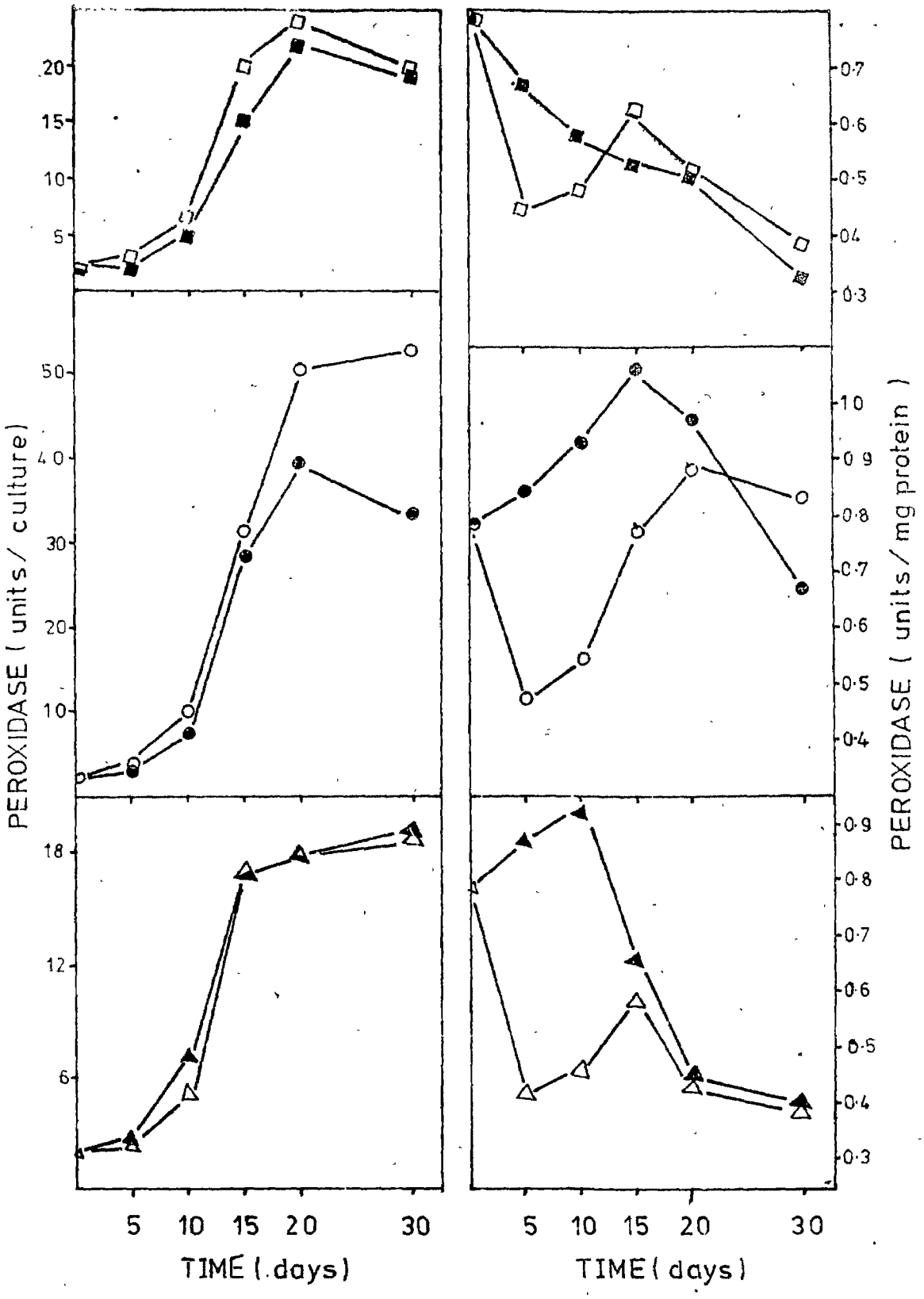

PEROXIDASE (units / culture)
PEROXIDASE (units / mg protein)
TIME (days)
TIME (days)
5 10 15 20 30
5 10 15 20 30

Fig. D-15

Table 25 : Effect of Light and Dark on IAA Oxidase Activity at Three 2,4-D Concentrations.
Inoculum : 300± 30 mg tissue by fresh weight on 30 ml MS medium supplemented with 0.2, 2.0 and 10.0 mg/l 2,4-D.
Incubation : 30 days at 26± 2°C.

Time (day)	2,4-D Conc. (mg/l)	IAA Oxidase*			
		In Light (1000 Lux)		In Dark	
		units/cult.	units/mg prot.	units/cult.	units/mg prot.
0	-	1.66	0.58	1.66	0.58
5	0.2	8.00	1.16	5.79	1.93
	2.0	8.05	0.96	5.95	1.81
	10.0	8.35	1.42	6.10	2.03
10	0.2	18.75	1.45	16.13	2.05
	2.0	20.07	1.34	19.55	2.18
	10.0	20.18	1.84	19.13	2.49
15	0.2	50.56	1.57	48.32	1.81
	2.0	69.13	2.15	53.36	1.86
	10.0	45.27	1.54	49.18	1.94
20	0.2	40.33	0.86	39.17	0.96
	2.0	41.79	0.72	40.14	0.92
	10.0	39.32	0.94	39.92	1.01
30	0.2	28.68	0.54	25.56	0.52
	2.0	35.65	0.56	32.91	0.55
	10.0	25.09	0.52	27.71	0.60

* One Unit of IAA Oxidase Activity = 1 mg of IAA destroyed/25 min at 37oC.

Fig. D-16. Total and specific activity of IAA oxidase as influenced by 2,4-D levels in presence and absence of light in callus cultures.

Treatments : MS medium with 0.2, 2.0 or 10.0 mg/l 2,4-D.

- □ - 0.2 mg/l 2,4-D - light,

- ■ - 0.2 " " - dark,

- O - 2.0 " " - light,

- ● - 2.0 " " - dark,

- △ - 10.0 " " - light, and

- ▲ - 10.0 " " - dark.

Experimental details as given in Table 25.

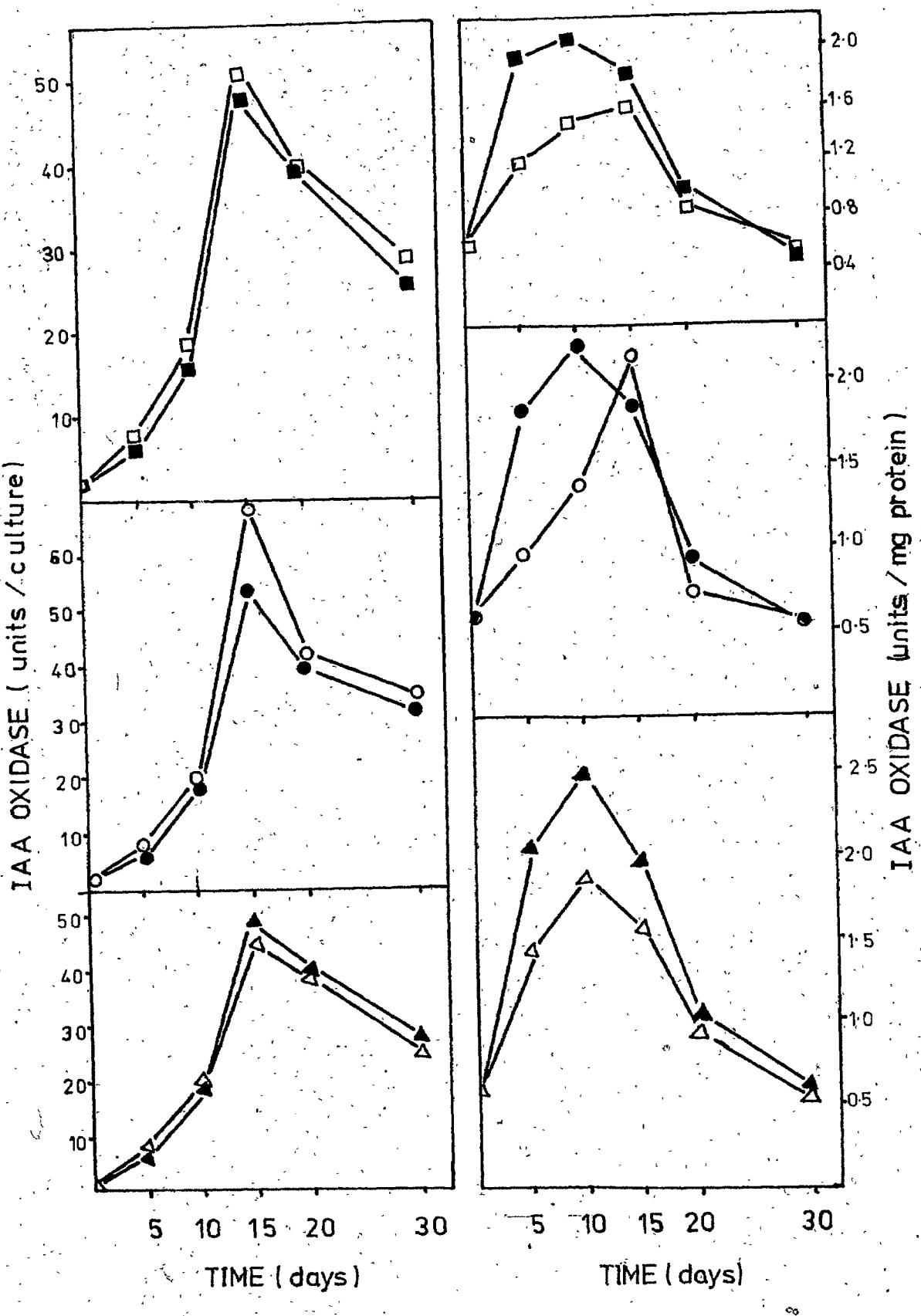

IAA OXIDASE (units / culture)
IAA OXIDASE (units / mg protein)
TIME (days)
TIME (days)
5
10
15
20
30

Fig. D-16

light at low and optimal 2,4-D levels. At higher 2,4-D concentration, more activity was recorded in dark grown tissues than in light grown ones. Maximum IAA oxidase activity was registered in tissues grown on 2.0 mg/l 2,4-D medium in light; while highest growth was supported by the same auxin concentration in the dark. IAA oxidase activity terminated earlier than peroxidase activity in all the cases. Also, after the enzyme reached a peak, there was sharp fall in the activity at all the 2,4-D levels.

Specific activity pattern of IAA oxidase activity showed different picture than peroxidase. There was steep raise in IAA oxidase in contrast to peroxidase activity where initial suppression in specific activity was observed. In case of 0.2 and 2.0 mg/l 2,4-D media, termination of IAA oxidase activity was recorded on day 15; while in case of 10.0 mg/l 2,4-D medium termination was observed 5 days early. At higher 2,4-D level, light and dark patterns were similar except higher specific activity was registered in dark. On the other hand, at low and optimal 2,4-D levels, specific activity showed decline in dark earlier than in light.

The developmental pattern of PAL and TAL as influenced in presence and absence of light at three 2,4-D concentrations is shown in Figs. D-17 and D-18 and presented in Tables 26 and 27 respectively. Light induction of both the enzyme activities was recorded in all the treatments; the PAL values being always higher than TAL. PAL and TAL activities were very low upto day 10; than they suddenly rose to reach their peak values on day 15 before declining sharply. The highest rate of PAL and TAL activities were recorded in 2.0 mg/l 2,4-D in light during 10-15 days period. while the highest polyphenol production was registered from 10-20 days. Thus, the polyphenols continued to accumulate even after the PAL and TAL activities terminated on day 15. A rough correspondence existed between the accumulation of phenolic compounds and the development of PAL and TAL activities at all 2,4-D levels during the course of culture for 15 days.

When the specific activities of PAL and TAL were compared with relative amount of phenolics produced, a close similarity was observed between them in light. In dark at higher 2,4-D level the developmental patterns

Table 27 : Effect of Light and Dark on TAL Activity at three 2,4-D Concentrations.

Inoculum : 300± 30 mg tissue by fresh weight on 30 ml MS medium supplemented with 0.2, 2.0 and 10.0 mg/l 2,4-D.

Incubation : 30 days at 26± 2°C.

Time (day)	2,4-D Conc. (mg/l)	T A L *			
		In Light (1000 Lux)		In Dark	
		Units/cult.	units/mg prot.	units/cult.	units/mg prot.
0	-	0.067	0.024	0.067	0.024
5	0.2	0.146	0.021	0.092	0.031
	2.0	0.245	0.029	0.084	0.028
	10.0	0.135	0.023	0.087	0.029
10	0.2	0.508	0.039	0.268	0.034
	2.0	0.707	0.038	0.276	0.035
	10.0	0.285	0.026	0.254	0.033
15	0.2	5.633	0.174	3.650	0.137
	2.0	7.625	0.189	4.609	0.161
	10.0	5.252	0.179	3.774	0.149
20	0.2	2.708	0.058	1.866	0.046
	2.0	5.056	0.087	2.307	0.057
	10.0	1.937	0.047	1.689	0.043
30	0.2	1.053	0.020	0.963	0.019
	2.0	1.590	0.025	1.036	0.021
	10.0	1.062	0.022	1.000	0.022

* One Unit of TAL Activity = formation of 10 umoles of p-Coumarate/hr at 37°C.

Fig. D-17. Effect of 2,4-D levels on the development of PAL activity (total and specific) in presence and absence of light.

Treatments : MS medium with 0.2, 2.0 or 10.0 mg/l 2,4-D.

-□- 0.2 mg/l 2,4-D - light,

-■- 0.2 " " - dark,

-○- 2.0 " " - light,

-●- 2.0 " " - dark,

-△- 10.0 " " - light, and

-▲- 10.0 " " - dark.

Experimental details as given in Table 26.

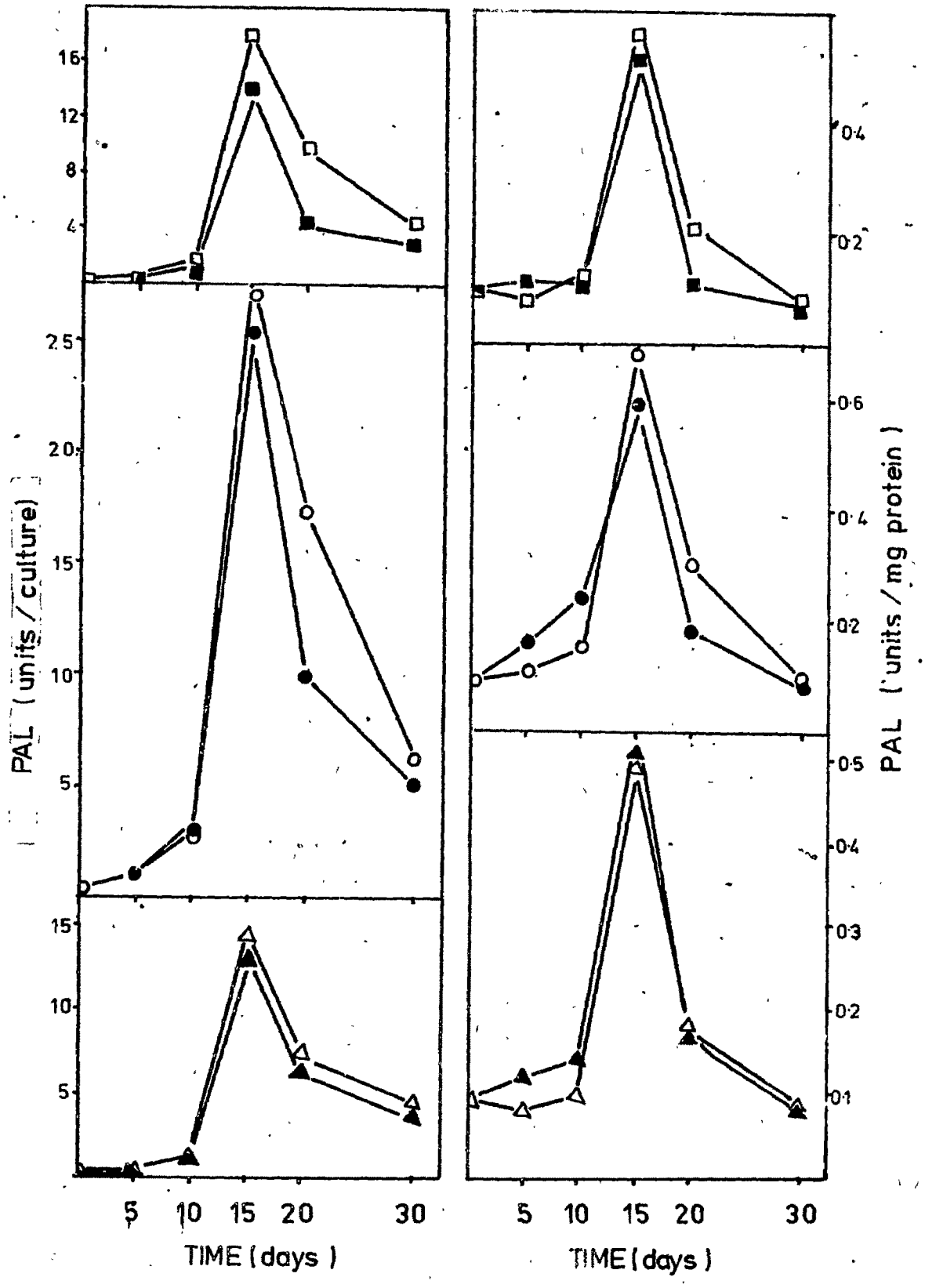

PAL (units / culture)
PAL (units / mg protein)
TIME (days)
TIME (days)
5
10
15
20
30

Fig. D-17

Table 26 : Effect of Light and Dark on PAL Activity in Crotalaria Callus Cultures Grown on Three 2,4-D Concentrations.
Inoculum : 300± 30 mg tissue by fresh weight on 30 ml MS medium supplemented with 0.2, 2.0 and 10.0 mg/l 2,4-D.
Incubation : 30 days at 26± 2°C.

Time (day)	2,4-D Conc. (mg/l)	PAL *			
		In Light (1000 Lux)		In Dark	
		units/cult	units/mg prot.	units/cult.	units/mg prot.
0	-	0.27	0.095	0.27	0.095
5	0.2	0.54	0.079	0.32	0.106
	2.0	0.93	0.111	0.90	0.157
	10.0	0.47	0.080	0.35	0.118
10	0.2	1.62	0.125	0.96	0.112
	2.0	2.76	0.152	3.07	0.242
	10.0	1.07	0.098	1.07	0.139
15	0.2	18.07	0.559	13.87	0.521
	2.0	27.45	0.680	25.36	0.593
	10.0	14.40	0.490	12.83	0.506
20	0.2	9.75	0.210	4.56	0.113
	2.0	17.19	0.298	9.89	0.178
	10.0	7.36	0.177	6.42	0.163
30	0.2	4.50	0.085	3.37	0.068
	2.0	6.27	0.098	5.18	0.084
	10.0	4.25	0.088	3.80	0.082

* One Unit of PAL Activity = formation of 10 umoles of Cinnamate/hr at 37°C.

Fig. D-18. The development of total and specific activity of TAL at various 2,4-D concentrations in presence and absence of light.

Treatments : MS medium supplemented with 0.2, 2.0 or 10.0 mg/l 2,4-D.

- □ - 0.2 mg/l 2,4-D - light,

- ■ - 0.2 " " - dark,

- o - 2.0 " " - light,

- ● - 2.0 " " - dark,

- Δ - 10.0 " " - light, and

- ▲ - 10.0 " " - dark.

Experimental details as given in Table 27.

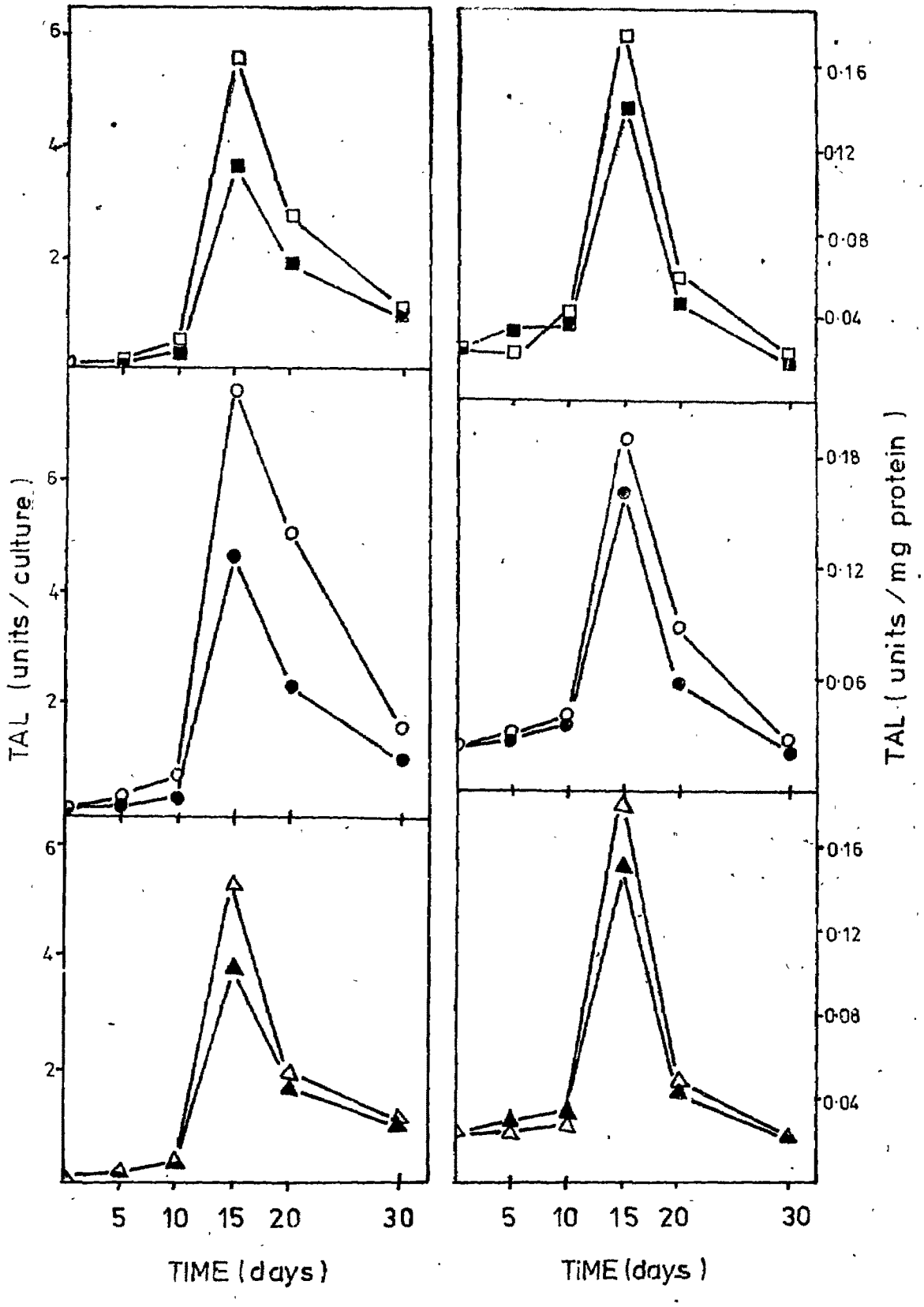

TAL (units / culture)
TAL (units / mg protein)
TIME (days)
TiME (days)
5
10
15
20
30
0·16
0·12
0·08
0·04
0·18
0·06

Fig. D-18

of PAL and TAL differed from that of polyphenol synthesis; the latter being continuously decreasing whereas the former reached a peak on day 15 before declining. At two other 2,4-D levels (0.2 and 2.0 mg/l) in dark, however, a rough correlation between PAL, TAL and polyphenol synthesis was observed.

3. NAA Effect on Growth, Polyphenol Synthesis and the Development of Peroxidase, IAA Oxidase, PAL and TAL in Presence and Absence of Light.

Another auxin, NAA, was tested for its influence on growth, polyphenol production and the development of peroxidase, IAA oxidase, PAL and TAL activities at three different concentrations both in light and in dark. Measured amount of callus pieces (300± 30 mg by fresh weight) were inoculated separately onto 30 ml MS medium supplemented with 0.2, 2.0 and 10.0 mg/l NAA.

One set of replicates was incubated under light (1000 Lux) and another set of replicates was kept in continuous dark. Both the sets were given identical conditions of temperature and humidity. A fixed number of replicate flasks was harvested at 5 days intervals

Table 28 : Effect of Light and Dark at Three NAA Concentrations on Growth of *Crotalaria* Callus Cultures*.

Inoculum : 300± 30 mg tissue by fresh weight on 30 ml MS medium supplemented with 0.2, 2.0 and 10.0 mg/l NAA.

Incubation : 30 days at 26± 2°C.

Time (day)	NAA Conc. (mg/l)	Growth			
		In Light (1000 Lux)		In Dark	
		Fresh wt. (mg)	Dry wt. (mg)	Fresh wt. (mg)	Dry wt. (mg)
0	-	300 (±30)	11.00 (±2.8)	300 (±30)	11.00 (±2.8)
5	0.2	411 (±28)	15.95 (±3.2)	415 (±38)	16.95 (±3.4)
	2.0	426 (±52)	16.26 (±4.8)	432 (±41)	17.00 (±4.1)
	10.0	451 (± 39)	17.18 (±3.8)	438 (±36)	17.08 (±5.0)
10	0.2	860 (±52)	43.00 (±4.9)	1006 (±43)	56.17 (±5.2)
	2.0	1294 (±68)	64.00 (±5.2)	1063 (±39)	54.37 (±6.1)
	10.0	917 (±49)	46.13 (±4.3)	1206 (±66)	58.00 (±4.9)
15	0.2	1520 (±71)	86.12 (±6.2)	1551 (±59)	86.81 (±7.2)
	2.0	2765 (±69)	107.36 (±7.1)	3080 (±68)	134.15 (±5.8)
	10.0	2383 (±57)	90.52 (±8.0)	2449 (±72)	107.36 (±8.0)
20	0.2	1695 (±80)	95.08 (±6.5)	2077 (±53)	105.00 (±6.9)
	2.0	3536 (±92)	148.63 (±7.9)	4513 (±48)	178.00 (±7.7)
	10.0	2902 (±75)	110.35 (±7.2)	3144 (±67)	138.82 (±8.2)
30	0.2	2025 (±43)	120.00 (±6.1)	2632 (±62)	128.51 (±6.1)
	2.0	5118 (±58)	211.87 (±5.6)	5383 (±78)	223.25 (±5.7)
	10.0	4471 (±62)	187.00 (±4.7)	4527 (±54)	191.32 (±4.9)

* Data represent average of six replicates.
Figures in the paranthesis represent standard errors.

Fig. D-19. NAA effect on growth (fresh and dry weights) in Crotalaria callus cultures in presence and absence of light.

Treatments : MS medium supplemented with 0.2, 2.0 or 10.0 mg/l NAA.

- □ - 0.2 mg/l NAA - light,

- ■ - 0.2 " " - dark,

- o - 2.0 " " - light,

- ● - 2.0 " " - dark,

- △ - 10.0 " " - light, and

- ▲ - 10.0 " " - dark.

Experimental details as given in Table 28.

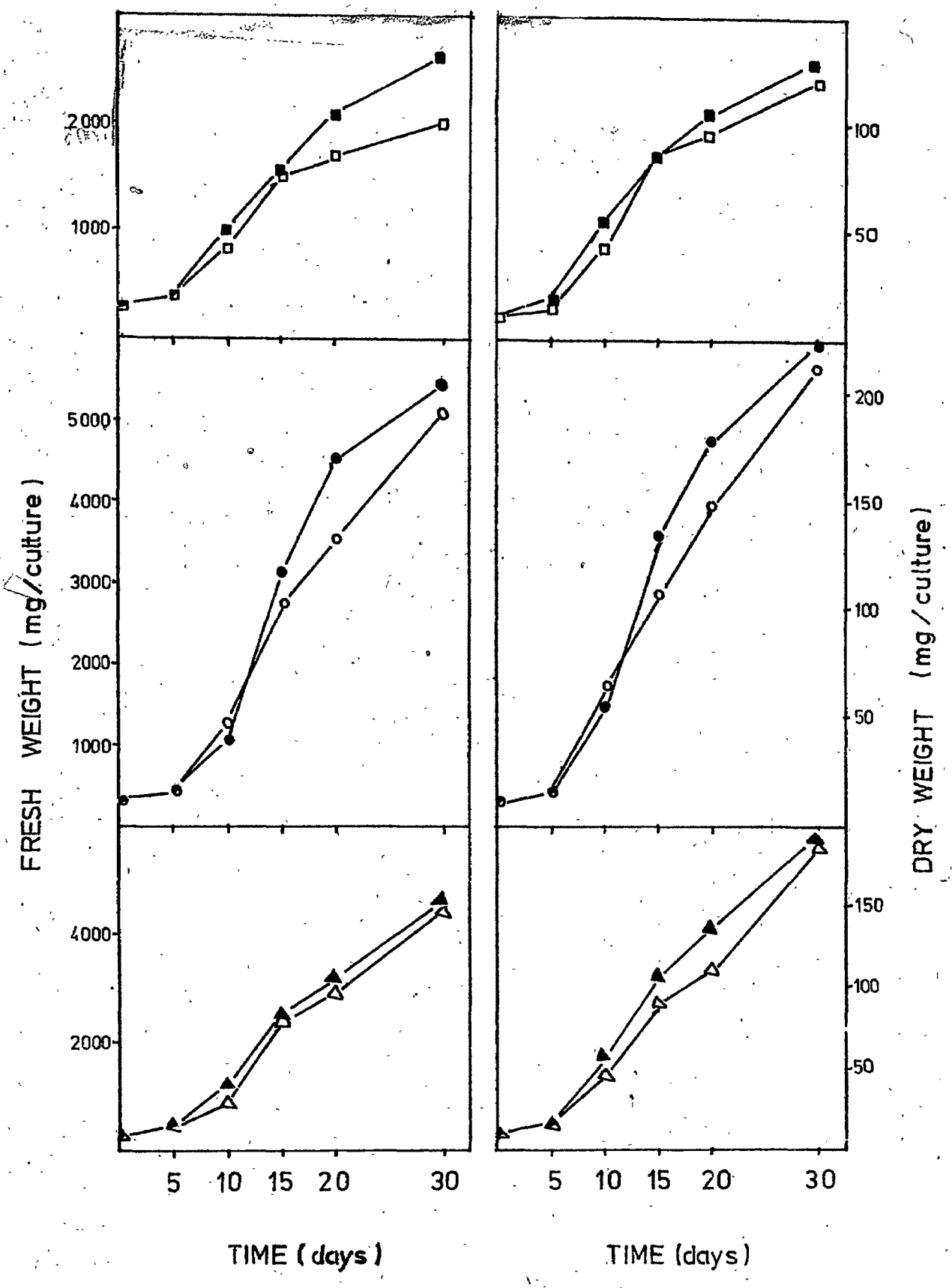

FRESH WEIGHT (mg/culture)
DRY WEIGHT (mg/culture)
2000
1000
5000
4000
3000
2000
1000
4000
2000
100
50
200
150
100
50
150
100
50
5 10 15 20 30
5 10 15 20 30
TIME (days)
TIME (days)

Fig. D-19

for the measurement of growth, estimation of phenolic materials and assaying peroxidase, IAA oxidase, PAL and TAL activities as described in Chapter II, Materials and Methods, 5A, 5B, 6A, 7A, 7B and 7C.

Like 2,4-D effect on growth, NAA also supported more growth in the dark (Fig. D-19 and Table 28). Growth was rapid after day 5 at all NAA concentrations and continued to increase till the end of culture period. However, at lower level of NAA (0.2 mg/l) increase in growth slowed down after day 15.

Light enhanced polyphenol production was observed at all the NAA levels (Fig. D-20 and Table 29). Maximum polyphenol synthesis was recorded at 2.0 mg/l NAA level in light. Further, supraoptimal level of NAA (10.0 mg/l) was found better than suboptimal level (0.2 mg/l). There was a steep rise in polyphenol synthesis after day 5 at all NAA concentrations both in light and in dark. Maximum rate of polyphenol synthesis was recorded from 5 - 15 day period; the increase in polyphenol production was slow between 15 - 20 day culture period at 2.0 and 10.0 mg/l NAA, after which again there was increase registering highest level on day 30. On the other hand, at low NAA

Table 29 : Effect of Light and Dark at three NAA Concentrations on Polyphenol Production.

Inoculum : 300± 30 mg tissue by fresh weight on 30 ml MS medium supplemented with 0.2, 2.0 and 10.0 mg/l NAA.

Incubation : 30 days at 26± 2°C.

Time	NAA Conc. (mg/l)	Polyphenols			
		In Light (1000 Lux)		In Dark	
		ug/culture	ug/100 mg dry wt.	ug/cultture	ug/100 mg dry wt.
0	-	88	800	88	800
5	0.2	151	950	140	825
	2.0	146	900	132	775
	10.0	150	875	137	800
10	0.2	495	1150	505	900
	2.0	672	1050	449	825
	10.0	438	950	443	850
15	0.2	1207	1400	1020	1175
	2.0	1288	1200	1174	875
	10.0	948	1050	966	900
20	0.2	927	975	919	825
	2.0	1338	900	1290	725
	10.0	1048	950	1041	750
30	0.2	900	750	835	650
	2.0	1748	825	1507	675
	10.0	1449	775	1339	700

Fig. D-20. Progressive changes in the synthesis of phenolic materials both per culture and on mean content at different NAA concentrations in presence and absence of light.

Treatments : MS medium with 0.2, 2.0 or 10.0 mg/l NAA.

- □ - 0.2 mg/l NAA - light,
- ■ - 0.2 " " - dark,
- ⊖ - 2.0 " " - light,
- ● - 2.0 " " - dark,
- △ - 10.0 " " - light, and
- ▲ - 10.00 " " - dark.

Other experimental details as given in Table 29.

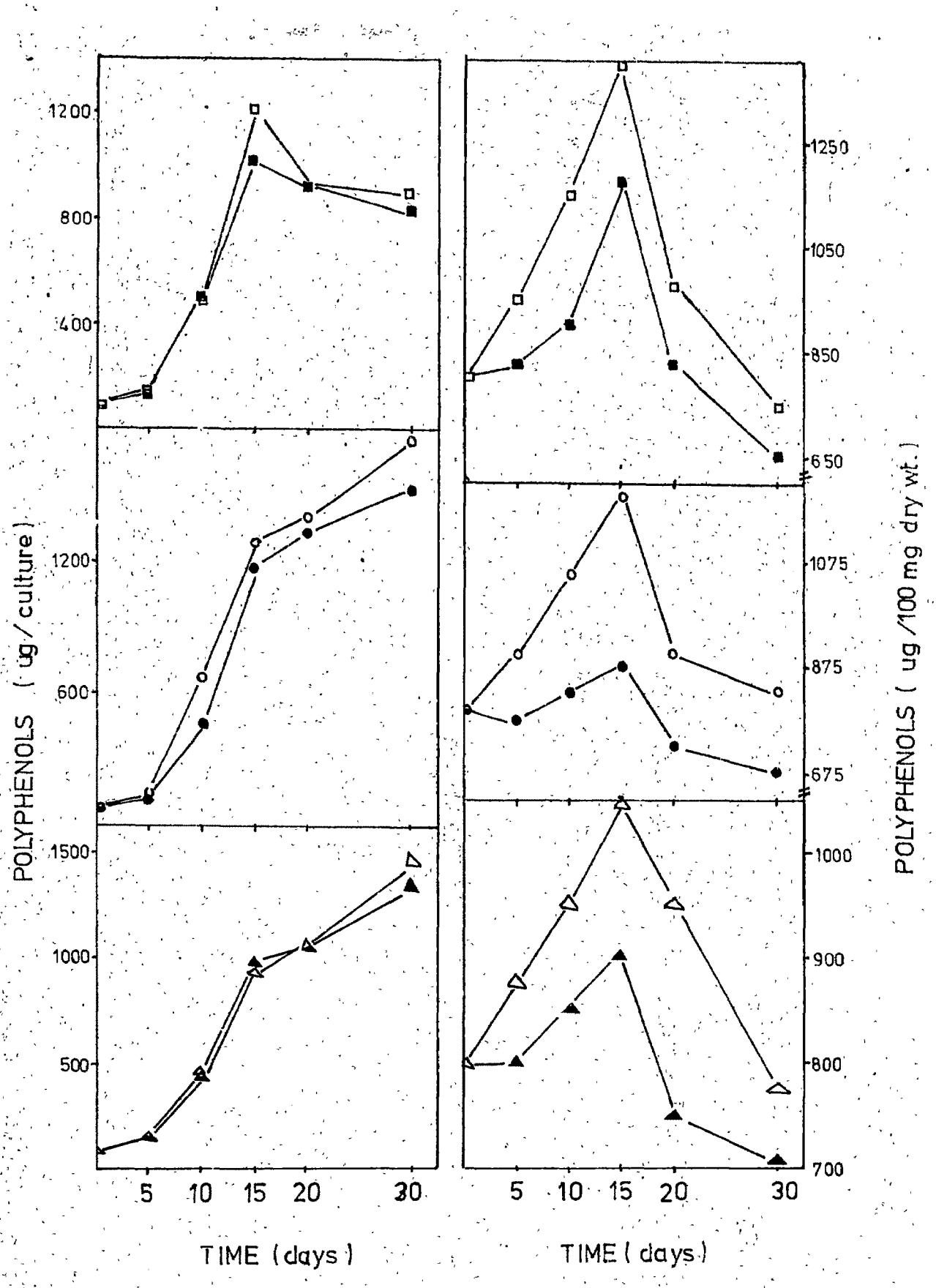

POLYPHENOLS (ug / culture)
1200
800
400
1200
600
1500
1000
500
5
10
15
20
30
TIME (days)
1250
1050
850
650
1075
875
675
1000
900
800
700
5
10
15
20
30
TIME (days)
POLYPHENOLS (ug /100 mg dry wt.)

Fig. D-20

concentration decline in polyphenol production set in on day 15 and continued till day 30.

Relative amount of polyphenols is presented in Fig. D-20 (Table 29). In light, sharp increase was observed from the beginning till day 15 followed by a sharp decline. In dark, the accumulation was low during initial 5 days after which it increased to reach a peak on day 15. Higher accumulation of phenolic materials was recorded on unit basis in the tissues grown on low NAA medium in light.

Fig. D-21 and Table 30 show the developmental pattern of peroxidase activity in light and in dark at various NAA concentrations. Clear induction of peroxidase activity by light was observed at all the NAA levels. Higher peroxidase activity was recorded in the tissues with higher polyphenol accumulation. 2.0 mg/l NAA in light supported maximum enzyme activity. Also, the maximum rate of the development of peroxidase was registered during 5 - 15 day period which corresponded with the maximum synthesis of polyphenols at various NAA levels. While at low NAA level there was decline in peroxidase activity after day 15, it continued to increase at 2.0 and 10.0 mg/l NAA as was the

Table 30 : Effect of Light and Dark on Peroxidase Activity in Crotalaria Callus Cultures Grown on Three NAA Concentrations.

Inoculum : 300± 30 mg tissue by fresh weight on 30 ml MS medium supplemented with 0.2, 2.0 and 10 mg/l NAA.

Incubation : 30 days at 26± 2°C.

Time (day)	NAA Conc. (mg/l)	Peroxidase*			
		In Light (1000 Lux)		In Dark	
		units/cult.	units/mg prot.	units/cult.	units/mg prot.
0	-	2.26	0.79	2.26	0.79
5	0.2	3.05	1.01	2.92	0.999
	2.0	2.88	0.82	2.78	0.91
	10.0	3.08	1.03	2.98	0.99
10	0.2	8.98	1.08	7.05	1.03
	2.0	9.78	1.04	6.98	1.00
	10.0	7.67	1.11	6.57	1.03
15	0.2	15.36	1.37	13.71	1.35
	2.0	18.72	1.41	16.86	1.39
	10.0	14.95	1.23	12.68	1.13
20	0.2	11.49	0.72	10.62	0.80
	2.0	20.56	1.09	18.81	1.15
	10.0	16.18	0.99	15.23	1.09
30	0.2	9.37	0.52	8.06	0.53
	2.0	24.56	1.00	19.53	0.88
	10.0	18.76	0.97	17.91	0.98

* One Unit of Peroxidase Activity = Change in OD of 10/min.

Fig. D-21. Progressive changes in peroxidase (total and specific) activity as influenced by different NAA concentrations in presence and absence of light.

Treatments : MS medium with 0.2, 2.0 or 10.0 mg/l NAA.

- □ - 0.2 mg/l NAA - light,
- ■ - 0.2 " " - dark,
- O - 2.0 " " - light,
- ● - 2.0 " " - dark,
- Δ - 10.0 " " - light, and
- ▲ - 10.0 " " - dark.

Experimental details as given in Table 30.

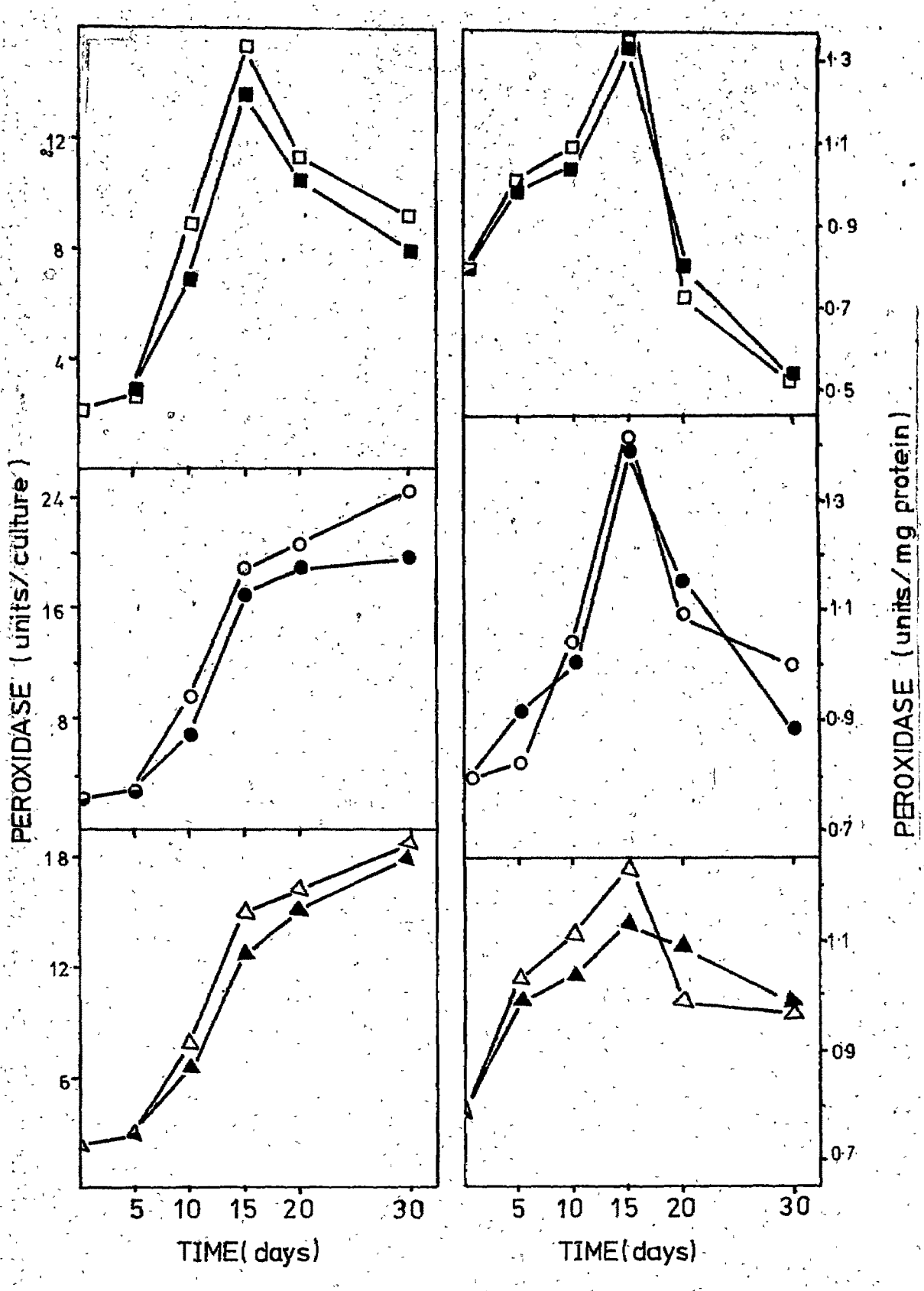

PEROXIDASE (units/culture)
PEROXIDASE (units/mg protein)
TIME(days)
TIME(days)
5 10 15 20 30
5 10 15 20 30

Fig. D-21

case with polyphenol accumulation. The patterns of specific activity of enzyme also showed close similarity with the accumulation of phenolic compounds on percentage basis at all the NAA concentrations tested.

The tissues grown on low NAA medium showed double peaks of IAA oxidase activity (Fig. D-22, Table 31). At 0.2 mg/l NAA, there was increase in IAA oxidase activity from the beginning reaching the first peak on day 15 followed by a fall and again rise in activity so as to attain the highest level on day 30. On the other hand, 2.0 and 10.0 mg/l NAA supported nearly linear increase in IAA oxidase activity both in light as well as in the dark. Induction of activity by light was observed at 0.2 and 2.0 mg/l NAA, while at higher NAA concentration higher levels of IAA oxidase activity was recorded in the dark throughout the culture period.

Specific activity of IAA oxidase activity showed first peak on day 15. After a fall in enzyme activity on day 20, there was noticed again a recovery in its activity. At low NAA level, the recovery was less as compared to 2.0 and 10.0 mg/l NAA and highest specific activity was registered on day 15 in contrast to 2.0 and 10.0 mg/l

Table 31 : Effect of Light and Dark on IAA Oxidase Activity at Three NAA Concentrations.

Inoculum : 300± 30 mg tissue by fresh weight on 30 ml MS medium supplemented with 0.2, 2.0 and 10.0 mg/l NAA.

Incubation : 30 days at 26± 2°C.

Time (day)	2,4-D Conc. (mg/l)	IAA Oxidase*			
		In Light (1000 Lux)		In Dark	
		units/cult.	units/mg prot.	units/cult.	units/mg prot.
0	-	1.66	0.58	1.66	0.58
5	0.2	7.05	2.34	6.95	2.36
	2.0	7.93	2.25	7.15	2.34
	10.0	7.62	2.55	7.15	2.38
10	0.2	20.85	2.50	17.85	2.62
	2.0	23.78	2.54	18.86	2.72
	10.0	18.96	2.75	21.25	3.34
15	0.2	35.45	3.16	31.32	3.07
	2.0	40.19	3.03	38.21	3.14
	10.0	39.38	3.24	42.32	3.78
20	0.2	29.02	1.83	24.79	1.88
	2.0	55.23	2.92	48.10	2.94
	10.0	47.50	2.90	49.10	3.52
30	0.2	38.21	2.12	35.38	2.31
	2.0	95.29	3.91	85.19	3.83
	10.0	72.17	3.73	81.48	4.50

* One Unit of IAA Oxidase Activity = 1 mg IAA destroyed/25 min at 37°C.

Fig. D-22. The development of IAA oxidase activity expressed per culture and per mg protein at various NAA levels in presence and absence of light.

Treatments : MS medium with 0.2, 2.0 or 10.0 mg/l NAA.

- □ - 0.2 mg/l NAA - light,

- ■ - 0.2 " " - dark,

- O - 2.0 " " - light,

- ● - 2.0 " " - dark,

- Δ - 10.0 " " - light, and

- ▲ - 10.0 " " - dark.

Experimental details as given in Table 31.

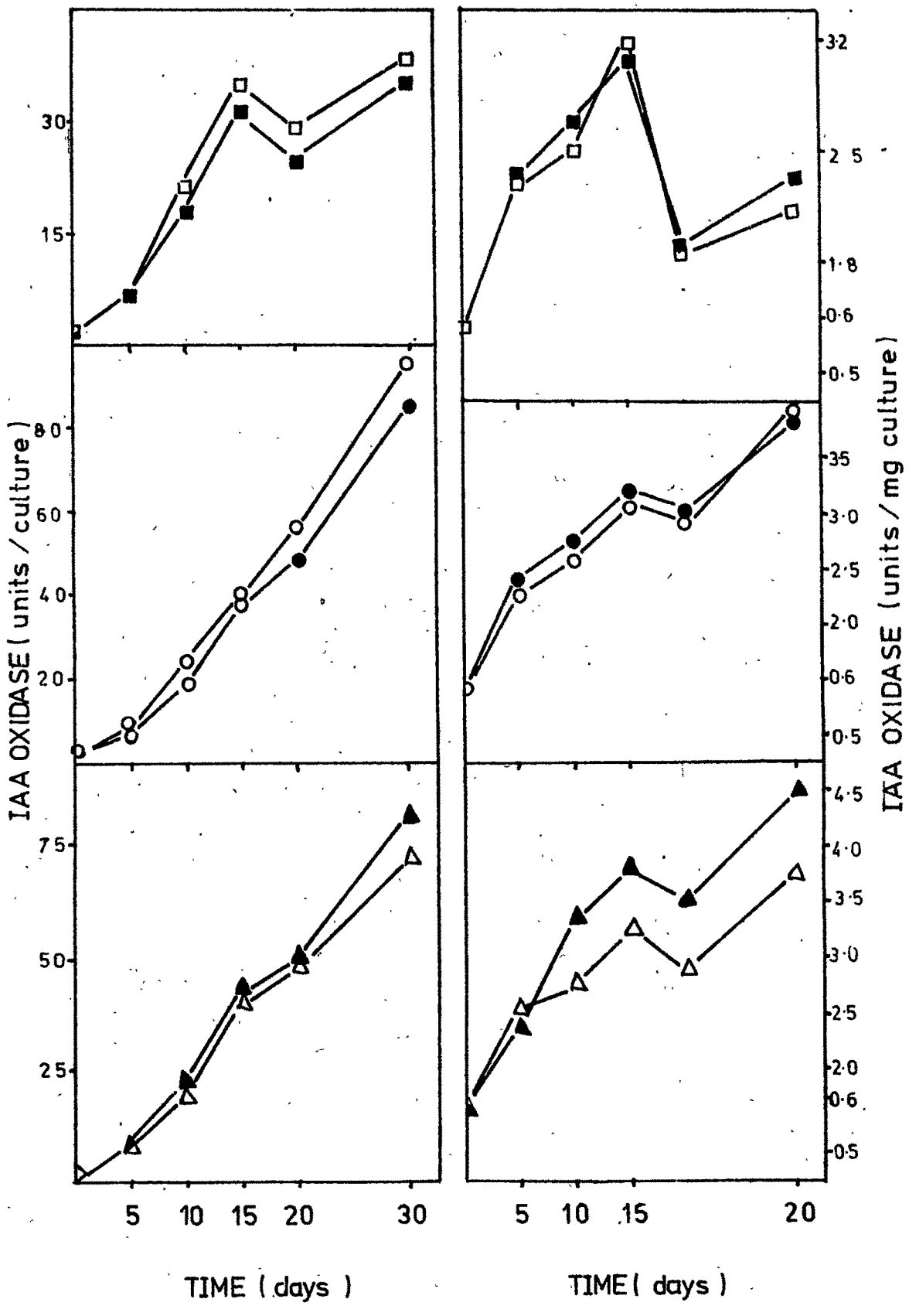

IAA OXIDASE (units / culture)
IAA OXIDASE (units / mg culture)
TIME (days)
TIME (days)
30
15
80
60
40
20
75
50
25
5
10
15
20
30
32
2 5
1·8
0·6
0·5
35
3·0
2·5
2·0
0·6
0·5
4·5
4·0
3·5
3·0
2·5
2·0
0·6
0·5

Fig. D-22

where higher activity was observed on day 30 both in light and in the dark. A rough correlation was recorded upto day 20 between the specific activity of IAA oxidase and relative amount of polyphenol synthesis at all the three NAA levels in light. In dark similar relationship was observed after an initial discrepancy.

PAL (Fig. D-23 and Table 32) and TAL (Fig. D-24 and Table 33) activities were low during the early part of culture period. A sharp increase was recorded both in light and in dark after day 10 reaching a peak value on day 15 followed by a sharp fall at all the three NAA levels. Higher PAL and TAL levels were recorded in the tissues grown on 2.0 mg/l NAA in light. Further, the maximum rate of both the enzyme activities were registered during 10 - 15 days culture period at various NAA levels. At low NAA, a rough similarity was observed between polyphenol synthesis and the development of PAL and TAL activities after day 10. The specific activity curves of PAL and TAL closely resembled with the curves of relative amount of phenolic compounds at various NAA levels in presence and absence of light.

Table 32 : Effect of Light and Dark on PAL Activity in Crotalaria Callus Cultures Grown on Three levels of NAA.

Inoculum : 300± 30 mg tissue by fresh weight on 30 ml MS medium supplemented with 0.2, 2.0 and 10 mg/l NAA.

Incubation : 30 days at 26± 2°C.

Time (day)	NAA Conc. (mg/l)	PAL *			
		In Light (1000 Lux)		In Dark	
		units/cult.	units/mg prot.	units/cult.	units/mg prot.
0	-	0.27	0.095	0.27	0.095
5	0.2	0.48	0.159	0.29	0.098
	2.0	0.75	0.213	0.80	0.262
	10.0	0.49	0.164	0.31	0.103
10	0.2	1.68	0.201	0.86	0.126
	2.0	2.36	0.252	2.12	0.306
	10.0	1.51	0.219	1.01	0.159
15	0.2	16.15	1.439	11.51	1.129
	2.0	25.18	1.899	21.08	1.735
	10.0	13.34	1.098	12.51	1.118
20	0.2	8.76	0.552	4.22	0.319
	2.0	15.32	0.809	12.16	0.745
	10.0	7.21	0.440	5.95	0.427
30	0.2	4.36	0.242	3.18	0.207
	2.0	5.76	0.236	4.44	0.200
	10.0	5.13	0.265	3.78	0.208

* One Unit of PAL Activity = formation of 10 umoles of cinnamate/hr at 37°C.

Fig. D-23. Progressive changes in the development of PAL (total and specific) activity at various NAA levels in presence and absence of light.

Treatments : MS medium supplemented with 0.2, 2.0 or 10.0 mg/l NAA.

- □ - 0.2 mg/l NAA - light,
- ■ - 0.2 " " - dark,
- ○ - 2.0 " " - light,
- ● - 2.0 " " - dark,
- △ - 10.0 " " - light, and
- ▲ - 10.0 " " - dark.

Experimental details as given in Table 32.

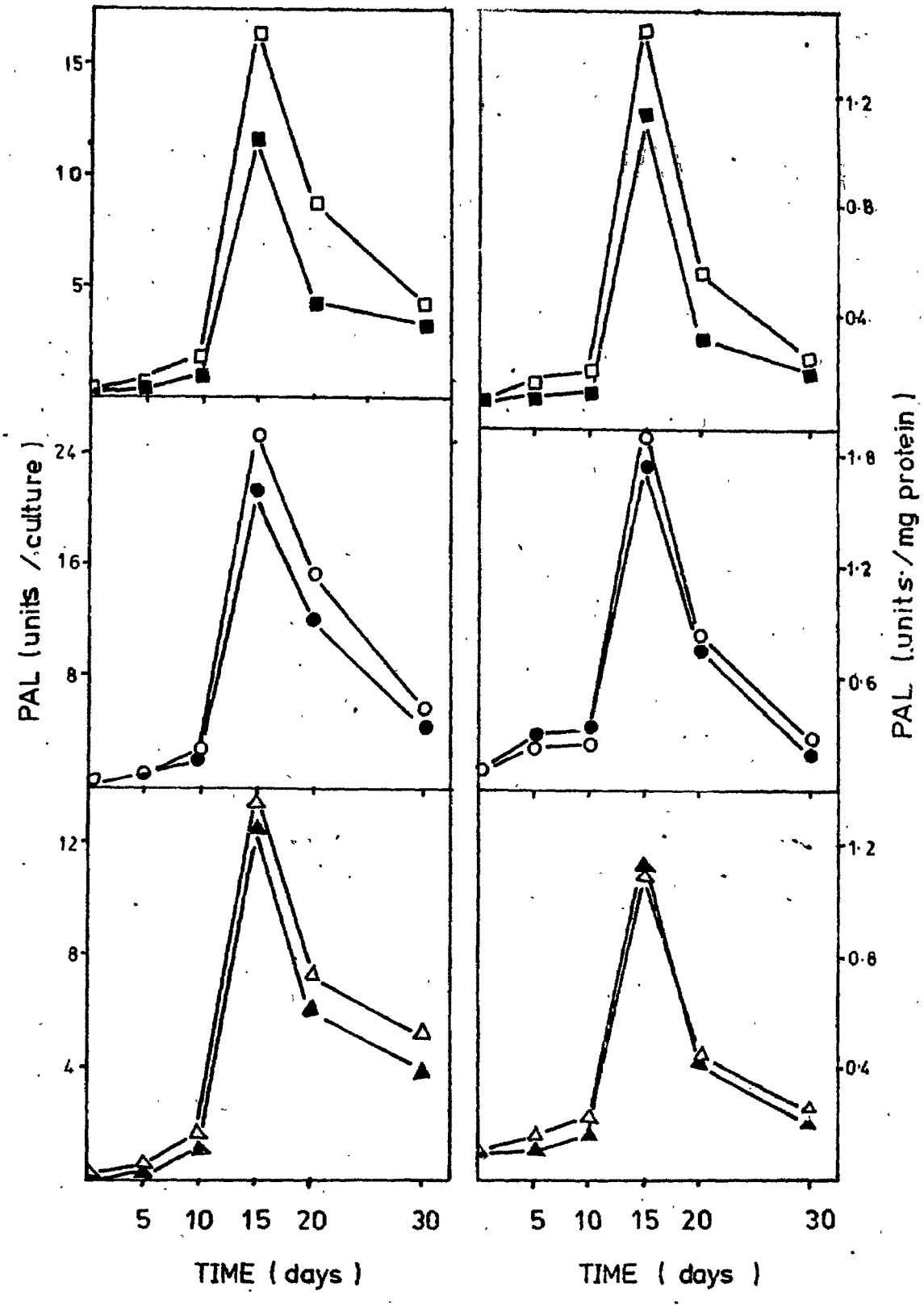

PAL (units /culture)
PAL (units/mg protein)
TIME (days)
TIME (days)
5 10 15 20 30
5 10 15 20 30

Fig. D-23

Table 33 : Effect of Light and Dark on TAL Activity of Three NAA Levels.

Inoculum : 300± 30 mg tissue by fresh weight on 30 ml MS medium supplemented with 0.2, 2.0 and 10.0 mg/l NAA.

Incubation : 30 days at 26± 2°C.

Time (day)	NAA Conc. (mg/l)	TAL *			
		In Light (1000 Lux)		IN Dark	
		units/cult.	units/mg prot.	units/cult.	units/mg prot.
0	-	0.067	0.024	0.067	0.024
5	0.2	0.12	0.039	0.080	0.027
	2.0	0.18	0.051	0.20	0.065
	10.0	0.13	0.044	0.080	0.027
10	0.2	0.43	0.051	0.22	0.032
	2.0	0.59	0.063	0.56	0.081
	10.0	0.39	0.056	0.29	0.046
15	0.2	3.59	0.319	2.74	0.268
	2.0	5.59	0.421	5.27	0.434
	10.0	3.35	0.275	3.13	0.279
20	0.2	2.43	0.153	1.17	0.088
	2.0	3.64	0.192	3.28	0.200
	10.0	2.06	0.126	1.65	0.118
30	0.2	1.03	0.057	0.94	0.061
	2.0	1.60	0.065	1.68	0.076
	10.0	1.22	0.063	0.96	0.053

* One Unit of TAL Activity = formation of 10 umoles of p-coumarate/ hr at 37°C.

Fig. D-24. Changes in total and specific activity of TAL as influenced by different NAA concentrations in presence and absence of light in Crotalaria callus cultures.

Treatments : MS medium with 0.2, 2.0 or 10.0 mg/l NAA.

- □ - 0.2 mg/l NAA - light,
- ■ - 0.2 " " - dark,
- O - 2.0 " " - light,
- ● - 2.0 " " - dark,
- Δ - 10.0 " " - light, and
- ▲ - 10.0 " " - dark.

Experimental details as given in Table 33.

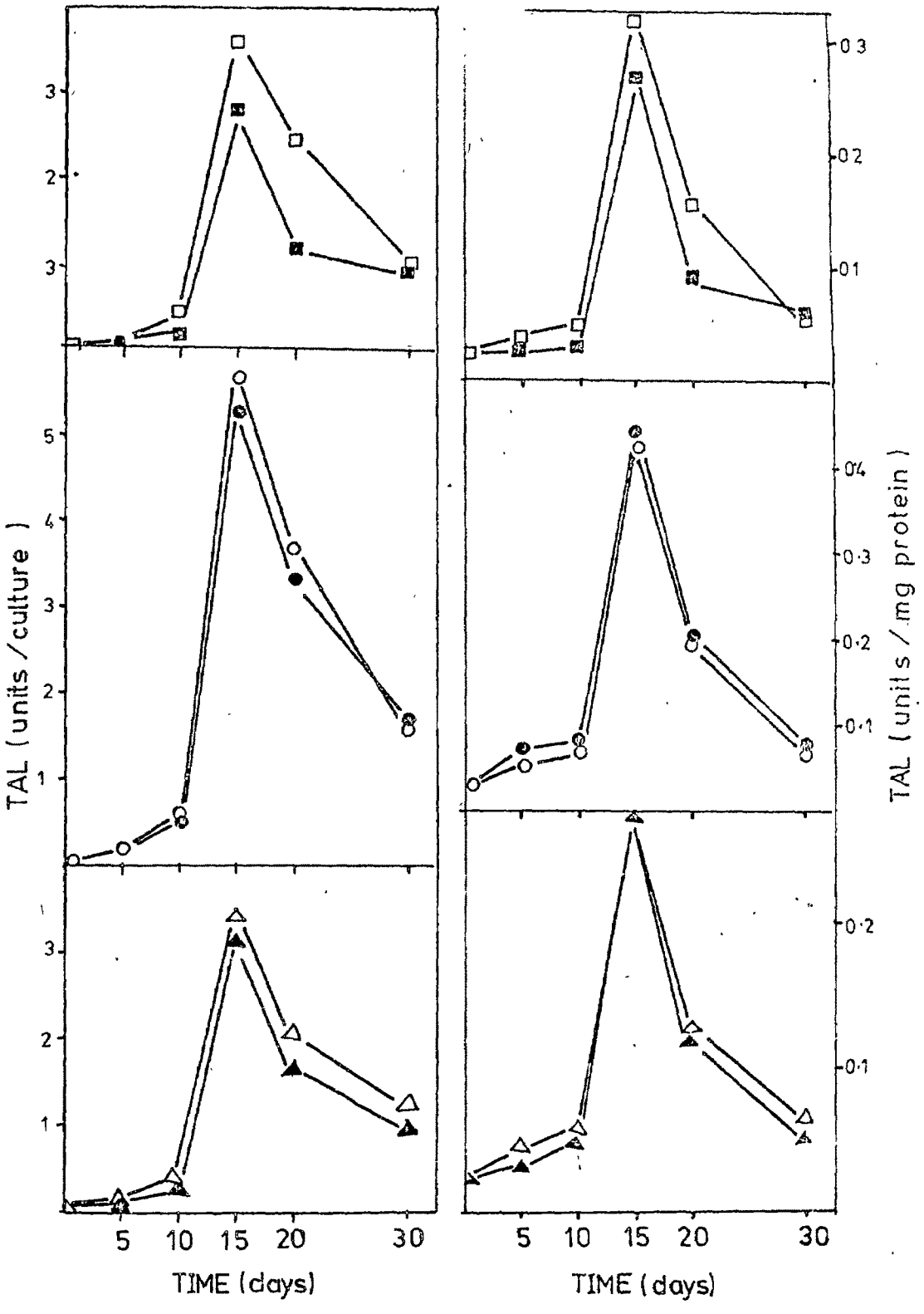

TAL (units / culture)
TAL (units / mg protein)
TIME (days)
TIME (days)
5
10
15
20
30

Fig. D-24

4. GA_3 Effect on Growth, Polyphenol Synthesis and the Development of Peroxidase, IAA Oxidase, PAL and TAL Activities in Presence and Absence of Light.

MS medium (Table 1, Chapter II) was supplemented with 1.0, 10.0 and 50.0 mg/l GA_3 in addition to 2.0 mg/l 2,4-D to examine its effect on growth, polyphenol production and the development of related enzymes. 300± 30 mg tissue by fresh weight was inoculated onto 30 ml medium in 150 ml Erlenmeyer flasks.

The flasks were incubated in light (1000 Lux) for 30 days. Another set of replicates was incubated in complete darkness. Both the sets were given identical conditions of temperature (26± 2°C) and humidity. A fixed number of replicate flasks was harvested every 5 days to determine growth, for the estimation of polyphenols and for assaying peroxidase, IAA oxidase, PAL and TAL activities.

The results showing the effect of light and complete darkness at various GA_3 concentrations on growth as measured by fresh and dry weights are presented in Table 34 and illustrated in Fig. D-25. At 1.0 mg/l GA_3 concentration growth values of the cultures exposed to light was low.

Table 34 : Effect of Light and Dark at Three GA_3 Concentrations on Growth of Crotalaria Callus Cultures*.

Inoculum : 300± 30 mg tissue by fresh weight on 30 ml MS medium supplemented with 1.0, 10.0 and 50.0 mg/l GA_3.

Incubation : 30 days at 26± 2°C.

Time (day)	GA_3 Conc. (mg/l)	Growth			
		In Light (1000 Lux)		In Dark	
		Fresh wt. (mg)	Dry wt. (mg)	Fresh wt. (mg)	Dry wt. (mg)
0	-	300 (±30)	11.00 (±2.8)	300 (±30)	11.00 (±2.8)
5	1.0	438 (±22)	18.11 (±1.7)	441 (±29)	18.16 (±3.1)
	10.0	462 (±28)	18.48 (±2.1)	458 (±35)	18.30 (±2.9)
	50.0	450 (±36)	17.98 (±3.2)	436 (±41)	17.35 (±3.6)
10	1.0	1258 (±58)	45.00 (±4.2)	1292 (±52)	46.36 (±4.2)
	10.0	1465 (±41)	55.07 (±3.8)	1428 (±57)	53.14 (±3.9)
	50.0	1489 (±39)	58.56 (±4.1)	1318 (±68)	52.00 (±5.8)
15	1.0	1786 (±68)	66.08 (±6.2)	1821 (±51)	68.92 (±6.2)
	10.0	3968 (±70)	148.96 (±6.6)	3896 (±62)	145.84 (±7.1)
	50.0	1671 (±54)	66.84 (±5.8)	1472 (±71)	56.27 (±6.9)
20	1.0	2928 (±72)	108.52 (±4.3)	3153 (±69)	111.32 (±5.7)
	10.0	5936 (±66)	210.47 (±6.8)	5681 (±80)	199.00 (±7.7)
	50.0	3213 (±52)	119.92 (±7.1)	2975 (±38)	112.98 (±6.1)
30	1.0	4792 (±51)	165.00 (±5.5)	4892 (±49)	170.12 (±5.6)
	10.0	6825 (±68)	242.38 (±4.8)	6790 (±55)	238.75 (±4.9)
	50.0	5017 (±49)	185.63 (±7.3)	4562 (±67)	168.48 (±7.5)

* Data represent average of six replicates.

Figures in the paranthesis represent standard errors.

Fig. D-25. Changes in growth (fresh and dry weights) as influenced by different GA_3 levels in presence and absence of light.

Treatments : MS medium supplemented with 1.0, 10.0 or 50 mg/l GA_3 in addition to 2.0 mg/l 2,4-D.

- □ - 1.0 mg/l GA_3 - light,
- ■ - 1.0 " " - dark,
- O - 10.0 " " - light,
- ● - 10.0 " " - dark,
- Δ - 50 " " - light, and
- ▲ - 50 " " - dark.

Experimental details as given in Table 34.

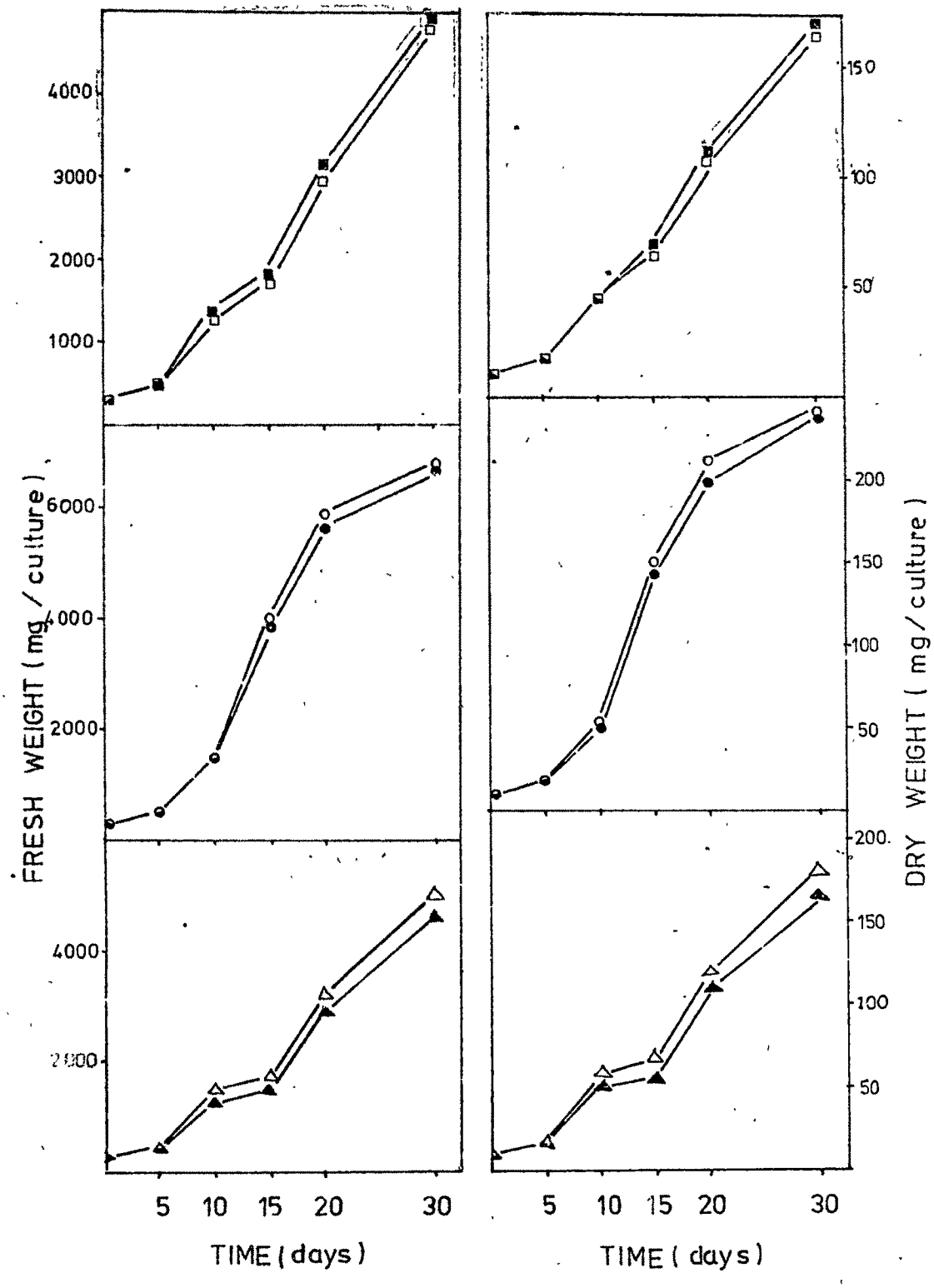

FRESH WEIGHT (mg / culture)
DRY WEIGHT (mg / culture)
4000
3000
2000
1000
6000
4000
2000
4000
150
100
50
200
150
100
50
200
150
100
50
5
10
15
20
30
TIME (days)
TIME (days)

Fig. D-25

However, the growth of the tissues subjected to 10.0 and 50.0 mg/l GA_3 level and exposed to light was higher than that of tissues grown in dark. This suggested the reversal of growth suppression in presence of light by low concentration of GA_3.

Accumulation of polyphenols as influenced by GA_3 concentrations in light and in dark is shown in Fig. D-26 and presented in Table 35. GA_3 at 10.0 mg/l concentration in light supported maximum polyphenol production. Further, low concentration of GA_3 was found better than high level for the accumulation of polyphenols. Light had promotory effect on polyphenol synthesis at low concentrations of GA_3 (1.0 and 10.0 mg/l); but marked suppression was recorded at higher GA_3 level in presence of light, more polyphenol synthesis being supported in the dark. Maximum rate of polyphenol synthesis was registered during 5 - 20 day period which roughly corresponded with maximum growth period. GA_3 at 10.0 mg/l in light supported more polyphenol production than 2,4-D alone in light.

The relative amount of phenolic materials present in the tissues at different GA_3 concentration in presence and in absence of light showed a peak on day 15 followed by a

Table 35 : Effect of Light and Dark at Three GA_3 Concentrations on Polyphenol Production.

Inoculum : 300± 30 mg tissue by fresh weight on 30 ml MS medium supplemented with 1.0, 10.0 and 50.0 mg/l GA_3.

Incubation : 30 days at 26± 2°C.

Time (day)	GA_3 Conc. (mg/l)	Polyphenols			
		In Light (1000 Lux)		In Dark	
		ug/culture	ug/100 mg dry wt.	ug/culture	ug/100 mg dry wt.
0	-	88	800	88	800
5	1.0	140	775	141	775
	10.0	157	850	146	800
	50.0	126	700	152	875
10	1.0	484	1075	394	850
	10.0	550	1000	505	950
	50.0	439	750	520	1000
15	1.0	942	1425	879	1275
	10.0	1558	1050	1494	1025
	50.0	719	1075	830	1475
20	1.0	1411	1300	1280	1150
	10.0	1896	900	1641	825
	50.0	899	750	1102	975
30	1.0	1775	1075	1616	950
	10.0	1999	825	1850	775
	50.0	1067	575	1305	775

Fig. D-26. Synthesis of phenolic compounds at different GA_3 concentrations in presence and absence of light.

Treatments : MS medium supplemented with 1.0, 10.0 or 50 mg/l GA_3 in addition to 2.0 mg/l 2,4-D.

- □ - 1.0 mg/l GA_3 - light,
- ■ - 1.0 " " - dark,
- O - 10.0 " " - light,
- ● - 10.0 " " - dark,
- Δ - 50 " " - light, and
- ▲ - 50 " " - dark.

Experimental details as given in Table 35.

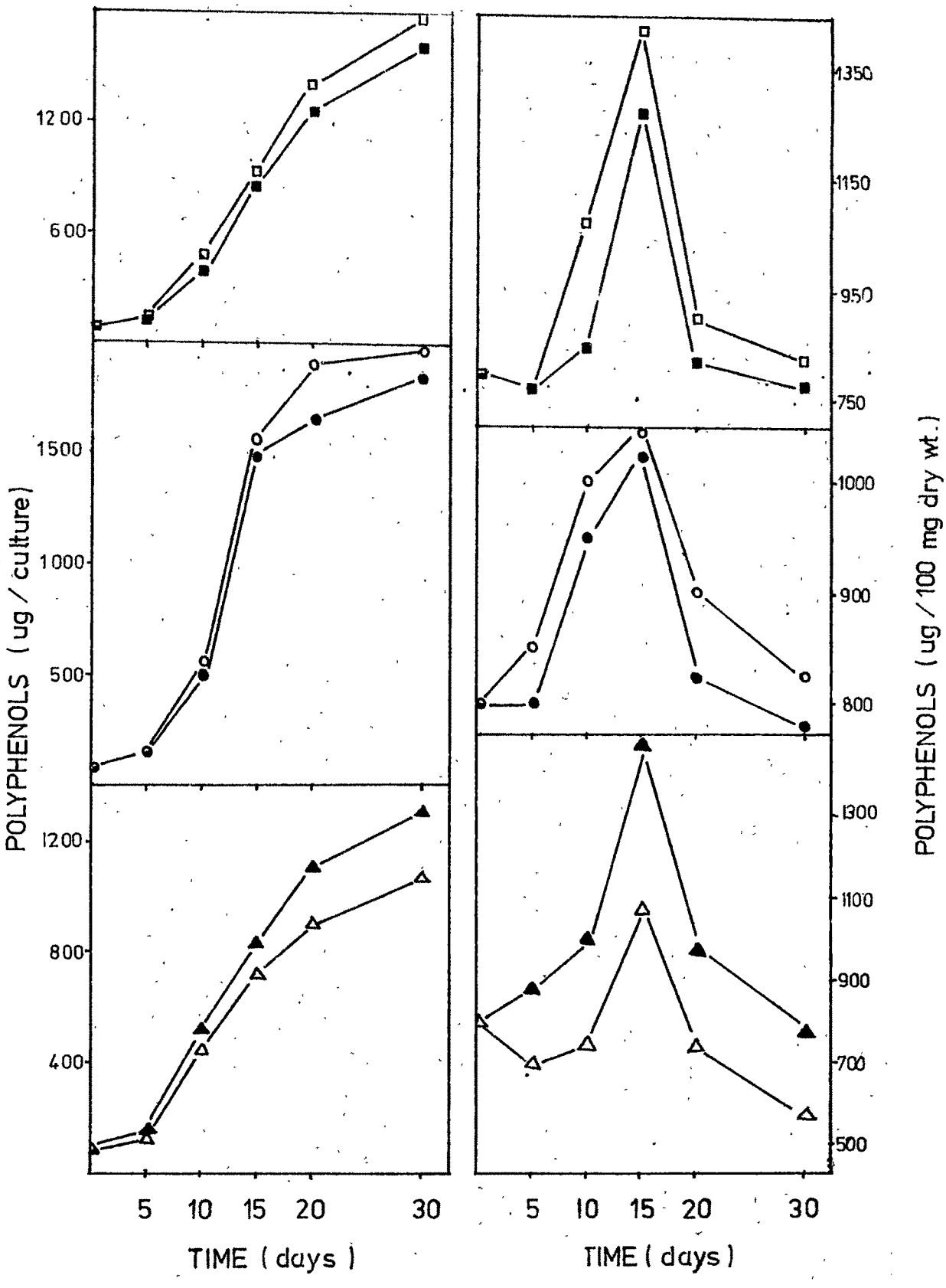

POLYPHENOLS (ug / culture)
POLYPHENOLS (ug / 100 mg dry wt.)
TIME (days)
TIME (days)
12 00
6 00
1500
1000
500
1200
800
400
1350
1150
950
750
1000
900
800
1300
1100
900
700
500
5
10
15
20
30
5
10
15
20
30

Fig. D - 26

sharp fall. At 1.0 and 10.0 mg/l GA_3 maximum level of phenolic compounds was recorded in the tissues grown in light, while at 50.0 mg/l it was registered in the dark grown tissues (Fig. D-26, Table 35). Among the hormones tested at different concentrations, GA_3 at 50.0 mg/l in presence of 2.0 mg/l 2,4-D in dark supported maximum polyphenol production (1.475%) on mean dry weight basis.

Light had stimulatory effect on peroxidase activity (Fig. D-27 and Table 36) at low GA_3 levels (1.0 and 10.0 mg/l). At high GA_3 level the promotory effect of light was suppressed and more enzyme activity was recorded in the tissues grown in total darkness. Phenolic content was the highest at 10.0 mg/l GA_3 in presence of light. Maximum rate of enzyme activity was recorded from 5 - 20 day at all GA_3 levels tested and both in presence and in absence of light, which closely corresponded with the rate of accumulation of phenolic compounds. The developmental pattern of peroxidase expressed in terms of specific activity showed a correlation after day 5 with that of the accumulation pattern of mean phenolic contents. However, there was some discripancy regarding the GA_3

Table 36 : Effect of Light and Dark on Peroxidase Activity in Crotalaria Callus Cultures Grown on Three GA_3 Levels.

Inoculum : 300± 30 mg tissue by fresh weight on 30 ml MS medium supplemented with 1.0, 10.0 and 50.0 mg/l GA_3.

Incubation : 30 days at 26± 2°C.

Time (day)	GA_3 Conc. (mg/l)	Peroxidase*			
		In Light (1000 Lux)		In Dark	
		units/cult.	units/mg prot.	units/cult.	units/mg prot.
0	-	2.26	0.79	2.26	0.79
5	1.0	2.57	0.38	2.05	0.31
	10.0	3.51	0.49	3.10	0.44
	50.0	2.83	0.42	3.00	0.42
10	1.0	6.99	0.65	5.72	0.62
	10.0	10.36	0.88	9.25	0.86
	50.0	6.52	0.82	7.26	0.71
15	1.0	18.04	0.78	16.05	0.79
	10.0	35.65	0.96	32.51	0.92
	50.0	11.28	0.91	12.53	0.88
20	1.0	24.61	0.63	21.36	0.64
	10.0	51.75	0.85	48.33	0.82
	50.0	15.32	0.78	16.38	0.73
30	1.0	26.87	0.48	22.98	0.45
	10.0	53.21	0.78	50.19	0.76
	50.0	18.19	0.53	19.21	0.49

* One Unit of Peroxidase Activity = change in OD of 10/min.

Fig. D-27. Progressive changes in total and specific activity of peroxidase at various GA_3 levels in presence and absence of light.

Treatments : MS medium supplemented with 1.0, 10.0 or 50 mg/l GA_3 in addition to 2.0 mg/l 2,4-D.

- □ - 1.0 mg/l GA_3 - light,
- ■ - 1.0 " " - dark,
- O - 10.0 " " - light,
- ● - 10.0 " " - dark,
- △ - 50 " " - light, and
- ▲ - 50 " " - dark.

Experimental details as given in Table 36.

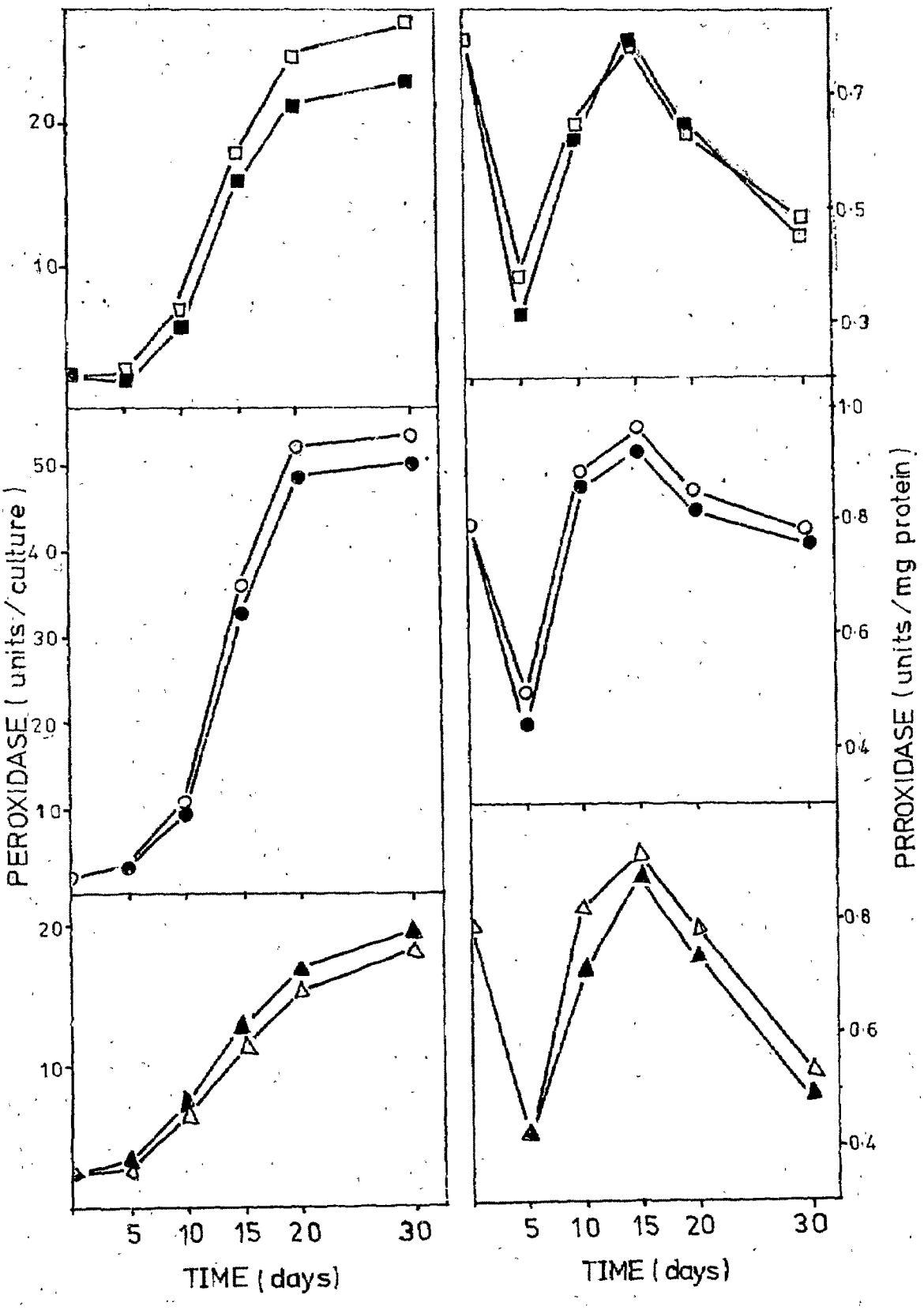

PEROXIDASE (units / culture)
PRROXIDASE (units / mg protein)
TIME (days)
TIME (days)
5
10
15
20
30
0·7
0·5
0·3
1·0
0·8
0·6
0·4

Fig. D-27

concentration. Thus, though maximum mean phenolic content was recorded at 50.0 mg/l GA_3 in absence of light, maximum specific activity of the enzyme was registered at 10.0 mg/l GA_3 in presence of light.

Fig. D-28 and Table 37 present the response of IAA oxidase to various levels of GA_3 in presence and in absence of light. Unlike peroxidase, IAA oxidase activity increased right from day 0. The peak in the enzyme activity was recorded on day 15 at all the GA_3 concentrations. Promotory effect of light was observed at 1.0 and 10.0 mg/l GA_3; while at 50.0 mg/l GA_3 higher activity was recorded in dark as was the case with peroxidase. 5 - 15 day period of increased IAA oxidase activity corresponded roughly with 5 - 15 day period of higher polyphenol accumulation. Further, though polyphenol continued to increase after day 15, IAA oxidase activity declined sharply. Specific activity patterns of IAA oxidase in presence and absence of light quite closely resembled the patterns of mean phenolic accumulation only at the high (50.0 mg/l) GA_3 level, the peaks being attained 5 days earlier at the optimal and suboptimal GA_3 levels.

As was the case with sucrose, 2,4-D and NAA, PAL

Table 37 : Effect of Light and Dark on IAA Oxidase Activity at Three GA_3 Concentrations.

Inoculum : 300± 30 mg tissue by fresh weight on 30 ml MS medium supplemented with 1.0, 10.0 and 50.0 mg/l GA_3.

Incubation : 30 days at 26± 2°C.

Time (day)	GA_3 Conc. (mg/l)	IAA Oxidase*			
		In Light (1000 Lux)		In Dark	
		units/cult.	units/mg prot.	units/cult.	units/mg prot.
0	-	1.66	0.58	1.66	0.58
5	1.0	13.52	2.00	11.05	1.69
	10.0	12.53	1.75	10.63	1.51
	50.0	11.61	1.72	12.59	1.80
10	1.0	36.38	3.38	32.19	3.51
	10.0	40.67	3.46	35.23	3.29
	50.0	31.50	3.96	34.68	3.42
15	1.0	72.51	3.13	64.32	3.18
	10.0	81.65	2.20	76.51	2.17
	50.0	69.36	5.60	78.42	5.53
20	1.0	48.12	1.23	41.17	1.24
	10.0	55.18	0.91	52.32	0.89
	50.0	39.18	1.99	46.79	2.09
30	1.0	41.39	0.74	33.89	0.67
	10.0	51.36	0.75	48.18	0.73
	50.0	31.22	0.90	38.28	0.98

* One Unit of IAA Oxidase Activity = 1 mg IAA destroyed/25 min at 37°C.

Fig. D-28. Progressive changes in the development of IAA oxidase (total and specific) activity at various GA_3 levels in presence and absence of light.

Treatments : MS medium with 1.0, 10.0 or 50 mg/l GA_3 in addition to 2.0 mg/l 2,4-D.

-□- 1.0 mg/l GA_3 - light,

-■- 1.0 " " - dark,

-O- 10.0 " " - light,

-●- 10.0 " " - dark,

-Δ- 50 " " - light, and

-▲- 50 " " - dark.

Experimental details as given in Table 37.

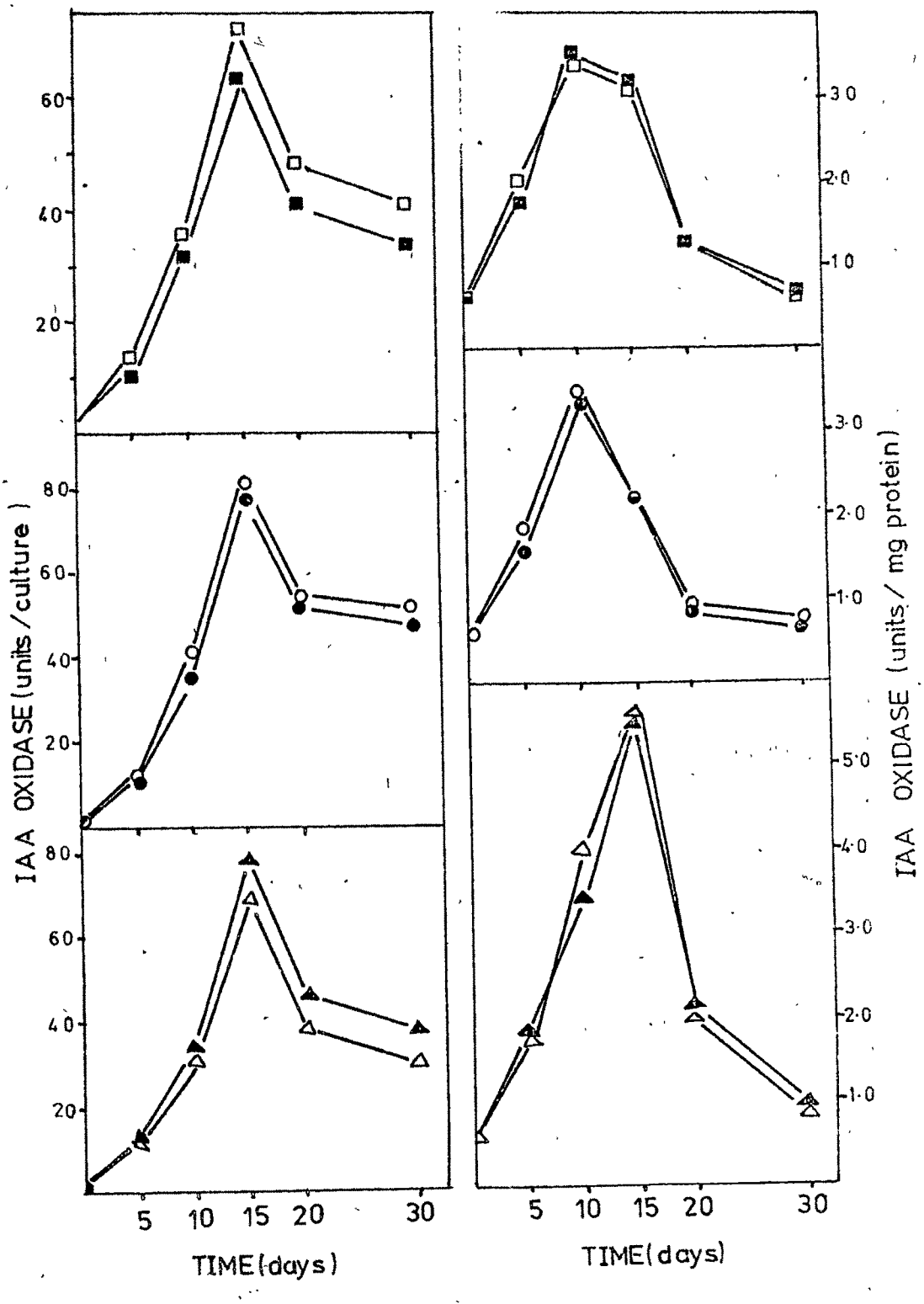

IAA OXIDASE (units / culture)
IAA OXIDASE (units / mg protein)
TIME (days)
TIME (days)
5
10
15
20
30

Fig. D-28

(Fig. D-29, Table 38) and TAL (Fig. D-30, Table 39) activities were low during the early culture period at all GA_3 levels tested. Between day 10 and 15 there was steep rise in both the enzyme activities followed by a sharp decline. Promotory effect of light was observed at 1.0 and 10.0 mg/l GA_3, while at higher GA_3 level more development of both the enzyme activities was recorded in the dark. This indicated that light induction of the said enzymes could be substituted by GA_3 at a higher dose.

Evaluation of the data on unit basis showed a close correlation of PAL and TAL with the accumulation of mean phenolic contents. However, in case of PAL at sub- and supraoptimal GA_3 concentrations discripancy was observed regarding the light effect. At supraoptimal GA_3, the specific activity of PAL was higher in dark than in light and at supraoptimal GA_3 level, the specific activity was higher in light than in dark. This is in contrast to mean phenolic compounds where higher level was recorded in light at suboptimal and in dark at supraoptimal level of GA_3. In case of TAL, this discripancy was observed only at higher GA_3 concentration.

Table 38 : Effect of Light and Dark on PAL Activity in Crotalaria Callus Cultures Grown on Three Levels of GA_3.

Inoculum : 300± 30 mg tissue by fresh weight on 30 ml MS medium supplemented with 1.0, 10.0 and 50.0 mg/l GA_3.

Incubation : 30 days at 26± 2°C.

Time (day)	GA_3 Conc. (mg/l)	PAL *			
		In Light (1000 Lux)		In Dark	
		units/cult.	units/mg prot.	units/cult.	units/mg prot.
0	-	0.27	0.095	0.27	0.095
5	1.0	0.92	0.136	0.76	0.116
	10.0	0.78	0.108	0.72	0.103
	50.0	0.75	0.111	0.79	0.113
10	1.0	2.52	0.234	1.96	0.214
	10.0	2.88	0.245	2.76	0.258
	50.0	2.43	0.305	2.92	0.288
15	1.0	18.89	0.816	17.21	0.852
	10.0	30.62	0.824	24.18	0.687
	50.0	15.35	1.239	16.87	1.189
20	1.0	13.21	0.338	11.52	0.347
	10.0	19.27	0.316	17.45	0.296
	50.0	10.61	0.539	12.15	0.543
30	1.0	6.29	0.112	7.10	0.140
	10.0	7.86	0.115	7.41	0.113
	50.0	4.83	0.140	6.03	0.155

* One Unit of PAL Activity = formation of 10 umoles of cinnamate/hr at 37°C.

Fig. D-29. The development of PAL activity as expressed per culture and per mg protein at different GA_3 levels in presence and absence of light.

Treatments : MS medium supplemented with 1.0, 10.0 or 50 mg/l GA_3 in addition to 2.0 mg/l 2,4-D.

-□- 1.0 mg/l GA_3 + light,

-■- 1.0 " " - dark,

-○- 10.0 " " - light,

-●- 10.0 " " - dark,

-△- 50 " " - light, and

-▲- 50 " " - dark.

Experimental details as given in Table 38.

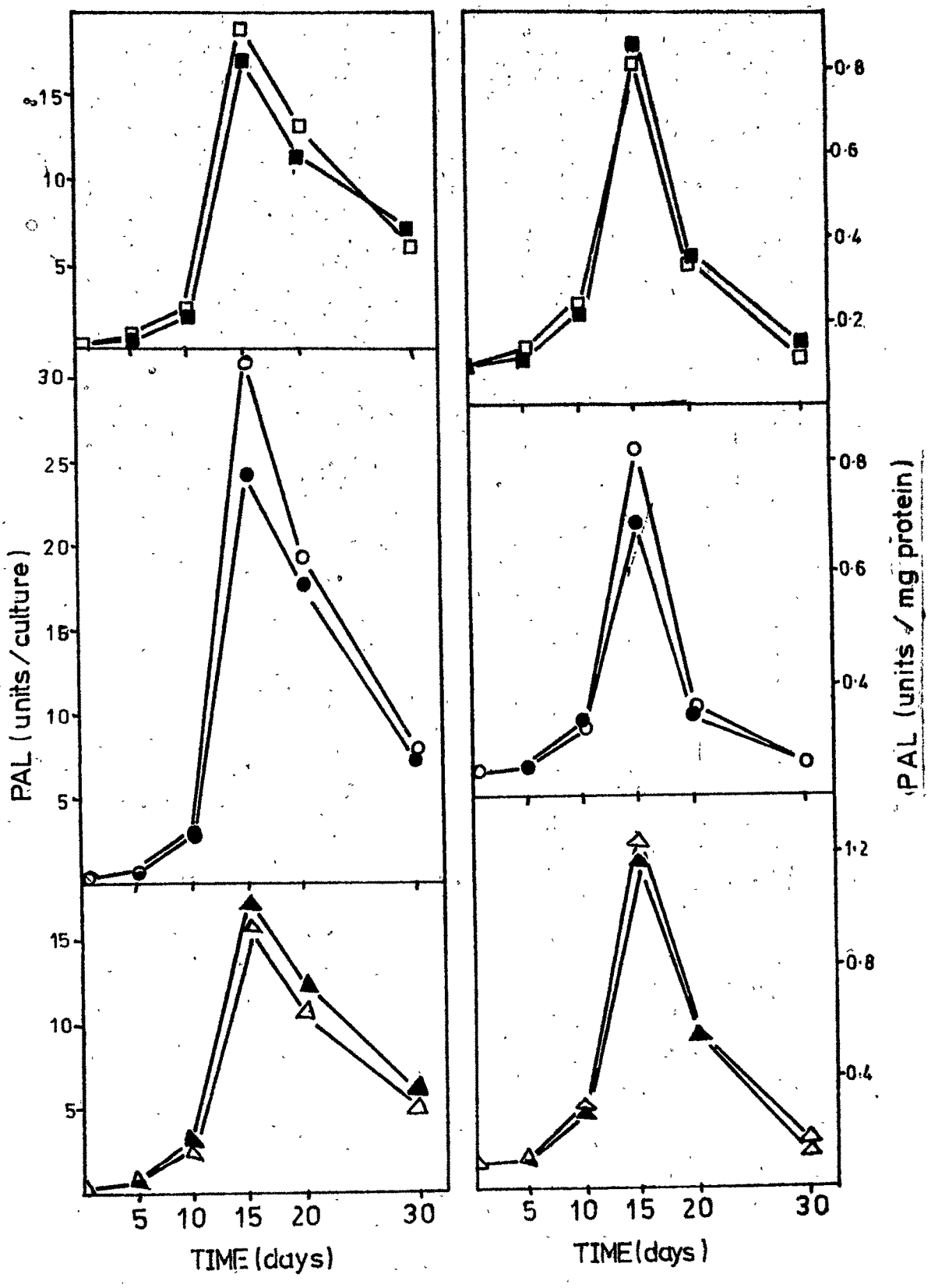

PAL (units/culture)
PAL (units / mg protein)
TIME (days)
TIME (days)
5
10
15
20
30
0·2
0·4
0·6
0·8
1·2

Fig. D-29

Table 39 : Effect of Light and Dark on TAL Activity at Three Levels of GA_3.

Inoculum : 300± 30 mg tissue by fresh weight on 30 ml MS medium supplemented with 1.0, 10.0 and 50.0 mg/l GA_3.

Incubation : 30 days at 26± 2°C.

Time (day)	GA_3 Conc. (mg/l)	TAL *			
		In Light (1000 Lux)		In Dark	
		units/cult.	units/mg prot.	units/cult.	units/mg prot.
0	-	0.067	0.024	0.067	0.024
5	1.0	0.292	0.043	0.217	0.033
	10.0	0.251	0.035	0.205	0.029
	50.0	0.227	0.034	0.255	0.036
10	1.0	0.742	0.069	0.560	0.061
	10.0	0.823	0.070	0.789	0.074
	50.0	0.812	0.102	0.859	0.084
15	1.0	5.253	0.227	4.529	0.224
	10.0	8.058	0.217	6.535	0.185
	50.0	4.796	0.387	4.962	0.349
20	1.0	3.669	0.094	3.490	0.105
	10.0	5.505	0.090	4.847	0.082
	50.0	3.121	0.159	3.472	0.155
30	1.0	2.029	0.036	1.928	0.038
	10.0	2.382	0.035	2.245	0.034
	50.0	1.380	0.040	1.945	0.049

* One Unit of TAL Activity = formation of 10 umoles of p-coumarate/hr at 37°C.

Fig. D-30. Changes in the development of TAL (total and specific) activity as influenced by various GA_3 levels in presence and absence of light.

Treatments : MS medium supplemented with 1.0, 10.0 or 50 mg/l GA_3 in addition to 2.0 mg/l 2,4-D.

- □ - 1.0 mg/l GA_3 - light,
- ■ - 1.0 " " - dark,
- O - 10.0 " " - light,
- ● - 10.0 " " - dark,
- Δ - 50 " " - light, and
- ▲ - 50 " " - dark.

Experimental details as given in Table 39.

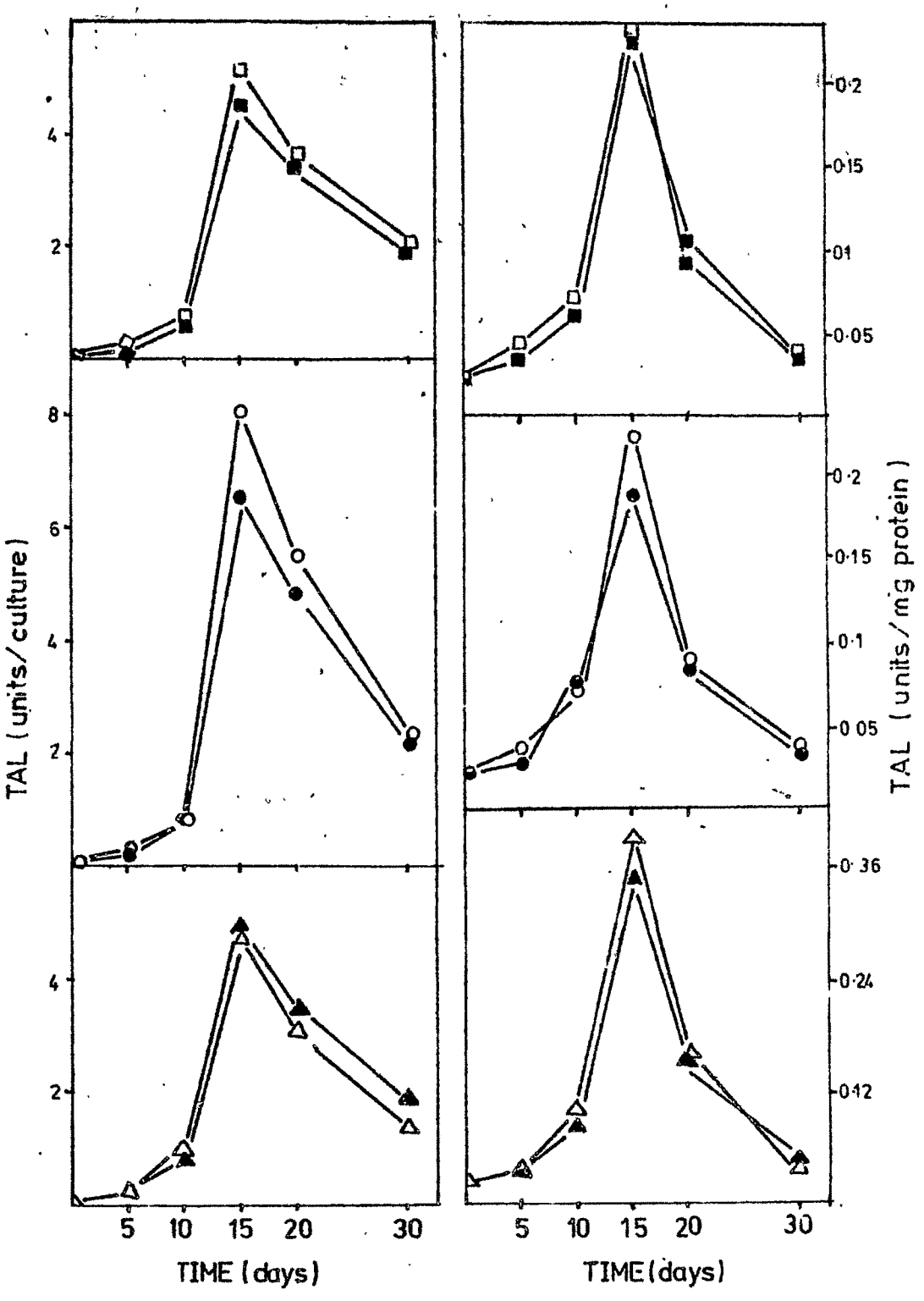

TAL (units/culture)
TAL (units/mg protein)
TIME (days)
TIME (days)
5
10
15
20
30
0·2
0·15
0·1
0·05
0·36
0·24
0·12

Fig. D-30

5. Effect of Kinetin on Growth, Polyphenol Accumulation and the Development of Peroxidase, IAA Oxidase, PAL and TAL Activities.

300± 30 mg tissue by fresh weight was inoculated onto 30 ml of MS medium containing 0.04, 0.4 or 4.0 mg/l kinetin in addition to 2.0 mg/l 2,4-D. The culture vessels were incubated for 30 days in light at 26± 2°C. A fixed number of replicate flasks was harvested every 5 days to determine growth, polyphenol content and for assaying the peroxidase, IAA oxidase, PAL and TAL enzymes.

Growth responses of the tissues at various kinetin concentrations are presented in Table 40 and illustrated in Fig. D-31 (fresh weights) and Fig. D-32 (dry weights). Increase in kinetin concentration in the medium led to the suppression of the growth. 5 to 20 days constituted the period of rapid growth phase at all the kinetin levels.

As shown in Fig. D-33 and Table 40, the maximum production of phenolic compounds was registered during the period of rapid growth (5 - 20 day) in all the concentrations tested. Further increase in polyphenol accumulation after day 20 was recorded only at low kinetin level,

Table 40 : Changes in Growth and Polyphenol Synthesis at Different Kinetin Concentrations.*

Inoculum : 300± 30 mg tissue by fresh weight on 30 ml MS medium supplemented with 0.04, 0.4 and 4.0 mg/l kinetin in addition to 2.0 mg/l 2,4-D.

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

Time (day)	0.04				0.4				4.0			
	Fresh wt. (mg)	Dry wt. (mg)	Polyphenols ug/cult.	Polyphenols ug/100 mg dry wt.	Fresh wt. (mg)	Dry wt. (mg)	Polyphenols ug/cult.	Polyphenols ug/100 mg dry wt.	Fresh wt. (mg)	Dry wt. (mg)	Polyphenols ug/cult.	Polyphenols ug/100 mg dry wt.
0	300 (±30)	11.00 (±2.8)	88	800	300 (±30)	11.00 (±2.8)	88	800	300 (±30)	11.00 (±2.8)	88	800
5	408 (±21)	15.00 (±1.9)	120	800	398 (±35)	14.85 (±3.1)	111	750	390 (±23)	15.08 (±4.1)	106	700
10	1151 (±62)	44.35 (±3.8)	377	850	1011 (±46)	38.15 (±4.2)	305	800	985 (±43)	37.21 (±5.1)	279	750
15	3225 (±56)	121.72 (±4.1)	1248	1025	2858 (±70)	109.32 (±6.5)	1038	950	2125 (±68)	80.82 (±6.1)	727	900
20	5236 (±81)	190.00 (±8.3)	1567	825	4885 (±38)	182.17 (±5.2)	1412	775	4061 (±61)	151.31 (±5.8)	1286	850
30	6018 (±73)	216.35 (±7.7)	1674	775	5611 (±25)	205.00 (±3.1)	1332	650	4835 (±42)	176.00 (±5.3)	1188	675

* Data represent average of six replicates.

Figures in the paranthesis represent standard errors.

Fig. D-31 to D-36. Changes in growth as measured by fresh (Fig. D-31) and dry (Fig. D-32) weights, the synthesis of phenolic materials (total - Fig. D-33 and relative amount - Fig. D-34) and the development of peroxidase activity (total - Fig. D-35 and specific - Fig. D-36 activity) as influenced by various kinetin concentrations.

Treatments : MS medium supplemented with 0.04, 0.4 or 4.0 mg/l kinetin in addition to 2.0 mg/l 2,4-D.

-□- 0.04 mg/l Kinetin,

-O- 0.4 " Kinetin, and

-Δ- 4.0 " Kinetin.

Experimental details as given in Table 40 and Table 41.

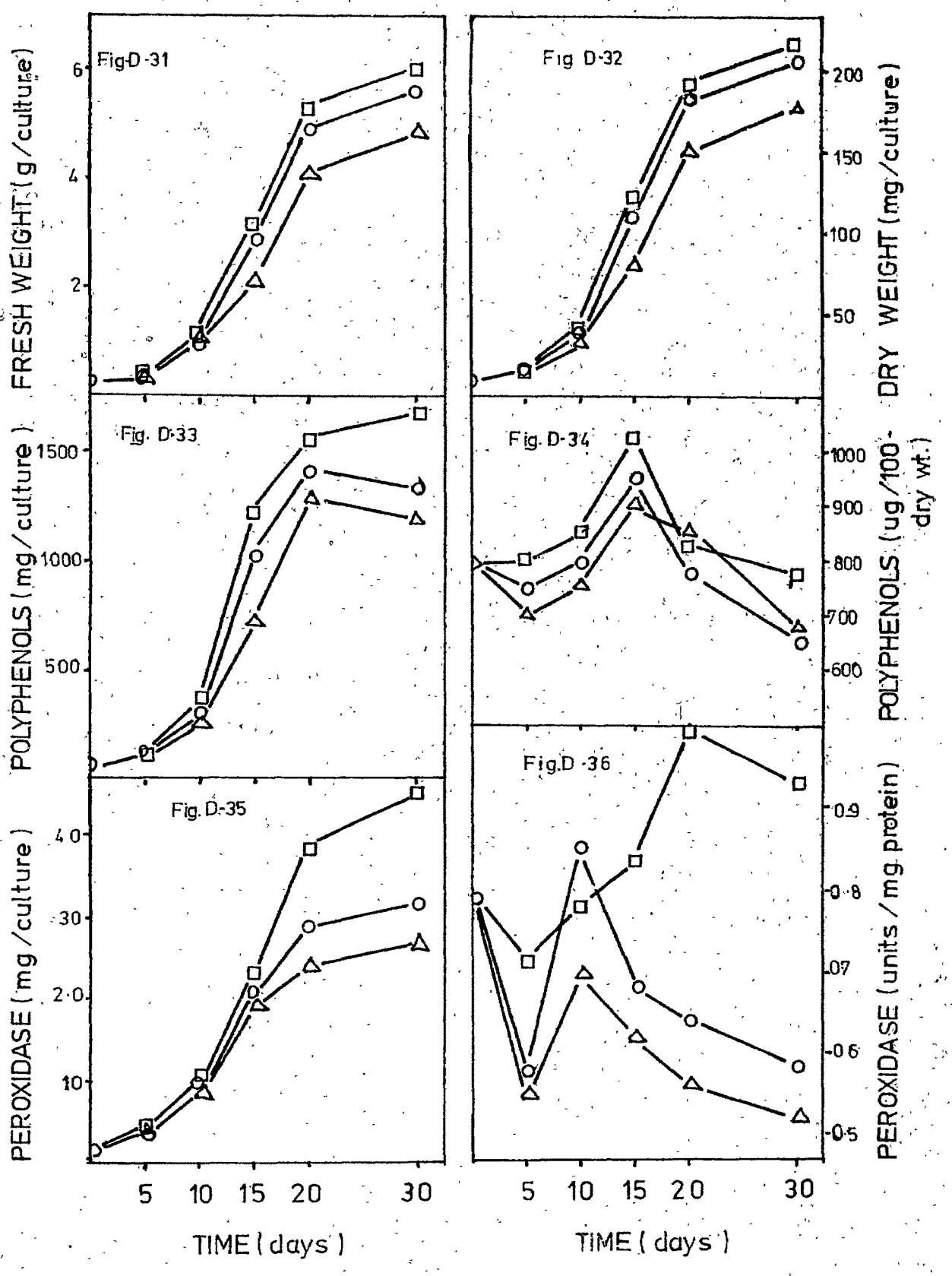


Fig-D-31
FRESH WEIGHT (g/culture)
Fig D-32
DRY WEIGHT (mg/culture)
Fig. D-33
POLYPHENOLS (mg/culture)
Fig. D-34
POLYPHENOLS (ug/100 dry wt.)
Fig. D-35
PEROXIDASE (mg/culture)
Fig.D-36
PEROXIDASE (units/mg protein)
TIME (days)
TIME (days)

while at 0.4 and 4.0 mg/l kinetin concentrations, a slight fall in the production of phenolic compounds was observed. Maximum synthesis of polyphenols was achieved at 0.04 mg/l kinetin level which also supported the maximum growth of the tissues. However, omission of kinetin from the medium supported more growth and also more polyphenol synthesis (see Figs. D-13 and D-14, Tables 22 and 23).

Mean phenolic contents of the tissues at various kinetin concentrations are illustrated in Fig. D-34 (Table 40). The initiation of polyphenol synthesis was much delayed in the tissues grown on high kinetin media. The peak value at all the kinetin levels was recorded on day 15, highest synthesis being in low kinetin medium.

The changes in the pattern of the development of peroxidase activity at different kinetin concentrations, illustrated in Fig. D-35 and presented in Table 41, showed that there was higher peroxidase activity in tissues with higher level of phenolic compounds. Thus, higher peroxidase activity was recorded at low kinetin level which also supported maximum polyphenol synthesis. Maximum rate of enzyme activity was registered during 5 - 20 days period

Table 41 : Influence of Kinetin on the Development of Peroxidase and IAA Oxidase Activities*.

Inoculum : 300± 30 mg tissue by fresh weight on 30 ml MS medium supplemented with 0.04, 0.4 and 4.0 mg/l kinetin in addition to 2.0 mg/l 2,4-D.

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

Time (day)	0.04				0.4				4.0			
	Peroxidase		IAA Oxidase		Peroxidase		IAA Oxidase		Peroxidase		IAA Oxidase	
	units/ cult.	units/mg protein	units/ cult.	units/mg protein	units/ cult.	units/ mg prot.	units/ cult.	units/ mg prot.	units/ cult.	units/ mg prot.	units/ cult.	units/ mg.prot.
0	2.26	0.79	1.66	0.58	2.26	0.79	1.66	0.58	2.26	0.79	1.66	0.58
5	4.51	0.71	7.95	1.26	3.98	0.58	8.60	1.26	3.82	0.55	9.23	1.34
10	9.80	0.78	18.62	1.48	8.75	0.85	20.28	1.99	8.50	0.70	22.51	1.88
15	23.42	0.83	55.18	1.96	20.63	0.68	57.65	1.90	19.40	0.62	55.36	1.76
20	38.00	1.00	59.23	1.57	28.56	0.64	60.13	1.35	23.81	0.56	63.52	1.50
30	44.66	0.92	36.78	0.76	30.84	0.58	38.33	0.72	26.52	0.52	42.18	0.82

One Unit of Peroxidase Activity = change in OD of 10/min.

One Unit of IAA Oxidase Activity = 1 mg IAA destroyed/25 min at 37°C.

of culture at all the kinetin levels which corresponded with rapid growth period and high rate of phenolic accumulation.

Specific activity pattern at different kinetin concentrations showed variations (Fig. D-36). After an initial suppression at all the kinetin levels there was sharp increase in specific activity. While the increase in specific activity of peroxidase continued upto day 20 at low kinetin concentration, it dropped sharply after day 10 at 0.4 and 4.0 mg/l kinetin levels.

IAA oxidase increased right from day 0 (Fig. D-37, Table 41). Unlike peroxidase and polyphenol accumulation, higher level of kinetin supported more activity. The peak in the enzyme activity was recorded on day 20 - after rapid rise for 15 days - at all the kinetin concentrations. At low kinetin level, specific activity of IAA oxidase (Fig. D-38) showed a peak on day 15 which corresponded with the peak of mean phenolic contents. On the other hand, at 0.4 and 4.0 mg/l kinetin levels, enzyme activity terminated 5 days earlier than the mean phenolic compounds.

PAL and TAL activities are illustrated in Figs. D-39

Table 42 : Effect of Kinetin on the Development of PAL and TAL Activities* in Crotalaria Callus Cultures.

Inoculum : 300± 30 mg tissue by fresh weight on 30 ml MS medium supplemented with 0.04, 0.4 and 4.0 mg/l kinetin in addition to 2.0 mg/l 2,4-D.

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

Time (day)	0.04				0.4				4.0			
	PAL		TAL		PAL		TAL		PAL		TAL	
	units/ cult.	units/ mg prot.	units/ cult.	units/ mg prot.	units/ cult.	units/ mg prot.	units/ cult.	units/ mg prot.	units/ cult.	units/ mg prot.	units/ cult.	units/ mg prot.
0	0.27	0.095	0.067	0.024	0.27	0.095	0.067	0.024	0.27	0.095	0.067	0.024
5	1.03	0.163	0.412	0.065	0.98	0.143	0.445	0.065	1.15	0.167	0.460	0.067
10	3.52	0.279	1.472	0.117	2.87	0.282	1.367	0.134	2.56	0.214	1.163	0.097
15	22.36	0.793	8.605	0.305	20.15	0.665	8.760	0.289	18.21	0.578	7.917	0.251
20	12.18	0.324	4.511	0.119	11.32	0.254	4.716	0 .105	9.17	0.217	3.821	0.090
30	5.96	0.123	2.128	0.044	4.85	0.092	1.940	0.037	4.23	0.082	2.014	0.039

One Unit of PAL Activity = formation of 10 umoles of cinnamate/hr at 37°C.

One Unit of TAL Activity = formation of 10 umoles of p-coumarate/hr at 37°C.

Fig. D-37 to D-42. Progressive changes in the development of total (Fig. D-37) and specific (Fig. D-38) activity of IAA oxidase, total (Fig. D-39) and specific (Fig. D-40) activity of PAL and total (Fig. D-41) and specific (Fig. D-42) activity of TAL at various kinetin concentrations.

Treatments : MS medium with 0.04, 0.4 or 4.0 mg/l kinetin in addition to 2.0 mg/l 2,4-D.

-□- 0.04 mg/l Kinetin,

-○- 0.04 " Kinetin, and

-△- 4.0 " Kinetin.

Experimental details as given in Table 41 and Table 42.

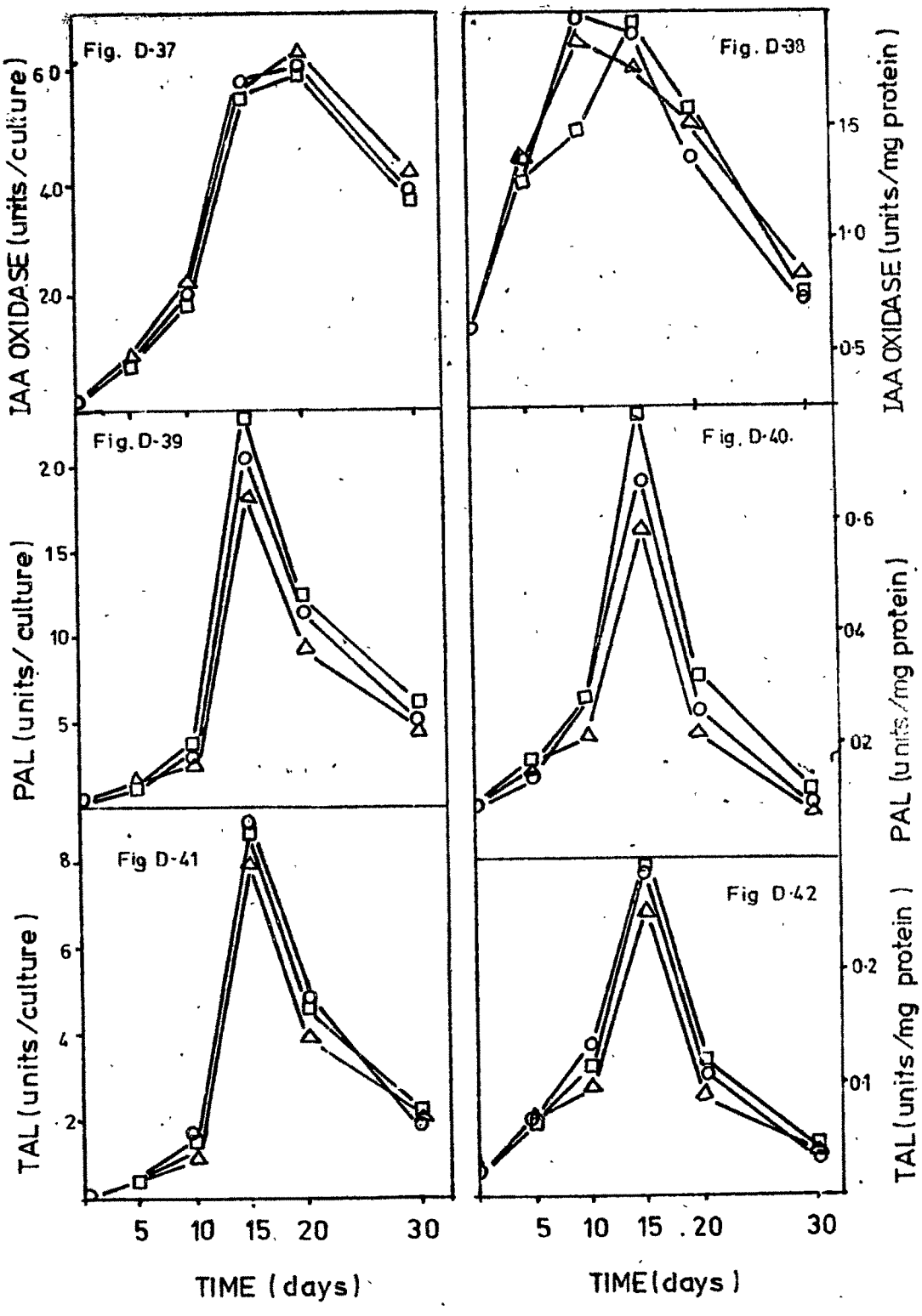


Fig. D-37
Fig. D-38
Fig. D-39
Fig. D-40
Fig D-41
Fig D-42
IAA OXIDASE (units/culture)
IAA OXIDASE (units/mg protein)
PAL (units/culture)
PAL (units/mg protein)
TAL (units/culture)
TAL (units/mg protein)
TIME (days)
TIME (days)

to D-42 and presented in Table 42. Higher level of kinetin suppressed the activity of both the enzymes. Maximum increase in PAL and TAL activities was recorded from 10 - 15 days. A sudden decline was observed at all the kinetin levels after day 15. The pattern of specific activity of PAL and TAL closely corresponded with the accumulation pattern of phenolic compounds expressed on relative amount basis, except for the initial five days.

6. Effect of Cycloheximide on Growth, Polyphenol Synthesis and the Development of Peroxidase, IAA Oxidase, PAL and TAL Activities.

A general protein inhibitor, cycloheximide, was tested to study the relative effect on growth, polyphenol accumulation and related enzyme activities. Weighed amount of callus pieces were inoculated separately on 30 ml MS medium supplemented with 10.0, 25.0 and 50.0 mg/l cycloheximide in addition to 2.0 mg/l 2,4-D.

The culture vessels were incubated in light at 26$\pm$ 2^{o}C for 30 days. A fixed number of replicate flasks was harvested at 5 days interval to determine fresh and dry weights, for the estimation of phenolic compounds

and for the assay of peroxidase, IAA oxidase, PAL and TAL as described in Chapter II, Materials and Methods, 5A, 5B, 6A, 7A, 7B and 7C.

The growth responses to various concentrations of cycloheximide are presented in Table 43 and shown in Figs. D-43 and D-44. Very strong inhibition of growth was recorded at all the cycloheximide levels over the control (2.0 mg/l 2,4-D medium which supported maximum 6772 mg fresh weight and 229 mg dry weight, Fig. D-13 and Table 22). The inhibitory effect increased with increase in cycloheximide concentration. Thus, 61.82, 81.96 and 89.84 % inhibition in fresh weights and 57.1, 76.54 and 84.20 % in dry weights were recorded in tissues grown on 10.0, 25.0 and 50.0 mg/l cycloheximide media respectively.

The accumulation of phenolic compounds, both in terms of per culture and on mean amount basis, was also inhibited at all the cycloheximide levels (Figs. D-45 and D-46 and Table 43), the inhibition being more marked at higher level (control medium supported 1889 µg/culture and 1100 µg/100 mg dry wt. phenolic materials as shown in Fig. D-14 and Table 23). In the case of relative amount of phenolic materials, there was pronounced suppression

Table 43 : Effect of Different Concentrations of Cycloheximide on Growth and Polyphenol Synthesis in Crotalaria Callus Cultures*.

Inoculum : 300± 30 mg tissue by fresh weight on 30 ml MS medium supplemented with 10.0, 25.0 and 50.0 mg/l cycloheximide in addition to 2.0 mg/l 2,4-D.

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

Time (day)	10.0				25.0				50.0			
	Fresh Wt. (mg)	Dry wt. (mg)	Polyphenols ug/cult.	Polyphenols ug/100 mg dry wt.	Fresh wt. (mg)	Dry wt. (mg)	Polyphenols ug/cult.	Polyphenols ug/100 mg dry wt.	Fresh wt. (mg)	Dry wt. (mg)	Polyphenols ug/cult.	Polyphenols ug/100 mg dry wt.
0	300 (±30)	11.00 (±2.8)	88	800	300 (±30)	11.00 (±2.8)	88	800	300 (±30)	11.00 (±2.8)	88	800
5	405 (±25)	19.08 (±2.5)	119	625	398 (±22)	15.32 (±2.7)	84	550	352 (±27)	14.90 (±2.4)	78	525
10	625 (±36)	29.80 (±3.2)	231	775	562 (±27)	26.12 (±3.1)	196	750	404 (±31)	20.92 (±2.5)	136	650
15	1511 (±51)	62.84 (±4.7)	456	725	835 (±50)	35.94 (±4.8)	252	700	552 (±43)	28.58 (±3.1)	178	625
20	2168 (±35)	85.22 (±5.1)	596	700	1036 (±70)	46.18 (±6.9)	289	625	607 (±35)	30.78 (±4.1)	169	550
30	2585 (±63)	98.19 (±5.8)	663	675	1221 (±56)	53.71 (±4.3)	309	575	688 (±42)	36.16 (±3.8)	163	450

* Data represent average of six replicates.

Figures in the paranthesis represent standard errors.

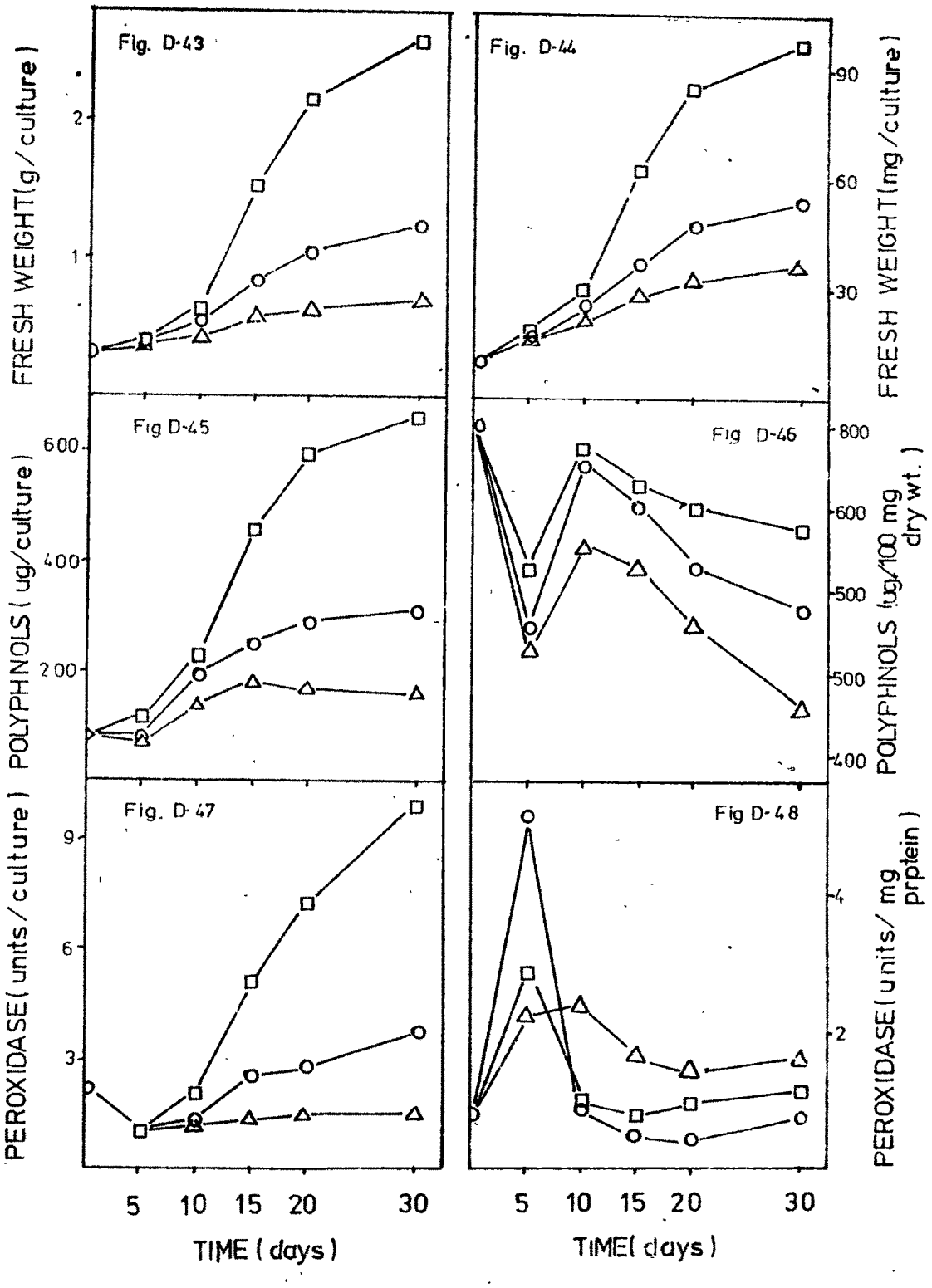


Fig. D-43
Fig. D-44
Fig D-45
Fig D-46
Fig. D-47
Fig D-48
FRESH WEIGHT(g/culture)
FRESH WEIGHT(mg/culture)
POLYPHNOLS (ug/culture)
POLYPHNOLS (ug/100 mg dry wt.)
PEROXIDASE(units/culture)
PEROXIDASE(units/mg prptein)
TIME (days)
TIME(days)
5 10 15 20 30
5 10 15 20 30

Fig. D-43 to D-48. Changes in growth as measured by fresh (Fig. D-43) and dry (Fig. D-44) weights, the synthesis of polyphenols (total - Fig. D-45 and mean content - Fig. D-46) and the development of peroxidase activity (total - Fig. D-47 and specific - Fig. D-48 activity) as influenced by different cycloheximide levels.

Treatments : MS medium supplemented with 10, 25 or 50 mg/l cycloheximide in addition to 2.0 mg/l 2,4-D.

-□- 10 mg/l Cycloheximide,

-○- 25 mg/l Cycloheximide, and

-△- 50 mg/l Cycloheximide.

Experimental details as given in Table 43 and Table 44.

of phenolic production on day 5, after which a recovery was made at all the cycloheximide concentrations upto day 10 followed by decline. The maximum phenolic compounds supported by 10.0, 25.0 and 50.0 mg/l cycloheximide were 663, 309 and 178 µg/culture which are 64.90, 83.64 and 90.57 % less than in the control medium (2.0 mg/l 2,4-D medium).

Total peroxidase activity (Fig. D-47, Table 44) was inhibited 97.33% over the control (control supported maximum 52.82 units/culture peroxidase activity, Fig. D-15 and Table 24) at high level of cycloheximide. At low cycloheximide, after an initial suppression, there was slow but steady increase in the enzyme activity till the end of culture period. However, the activity was 81.41% less as compared with the control.

Evaluation of the data on specific activity basis (Fig. D-48) showed initial induction at all the cycloheximide level followed by a sharp decline. Further, specific activity of peroxidase maintained higher level over the control (0.88 units/mg protein and 0.47 units/mg protein were the highest and lowest values for peroxidase specific activity supported by control medium as shown in

Table 44 : Changes in Peroxidase and IAA Oxidase Activities at Different Cycloheximide Levels.

Inoculum : 300± mg tissue by fresh weight on 30 ml MS medium supplemented with 10.0, 25.0 and 50.0 mg/l cycloheximide in addition to 2.0 mg/l 2,4-D.

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

Time (day)	10.0				25.0				50.0			
	Peroxidase		IAA Oxidase		Peroxidase		IAA Oxidase		Peroxidase		IAA Oxidase	
	units/ cult.	units/ mg prot.	units/ cult.	units/ mg prot.	units/ cult.	units/ mg prot.	units/ cult.	units/ mg prot.	units/ cult.	units/ mg prot.	units/ cult.	units/ mg prot.
0	2.26	0.79	1.66	0.58	2.26	0.79	1.66	0.58	2.26	0.79	1.66	0.58
5	1.10	3.85	1.92	7.30	1.06	5.09	2.23	10.66	0.99	2.26	2.00	4.54
10	2.09	1.01	6.40	3.15	1.42	0.86	6.25	3.78	1.25	2.39	3.25	6.19
15	5.04	0.78	4.53	0.69	2.48	0.49	2.25	0.45	1.32	1.67	1.65	2.08
20	7.24	0.98	3.73	0.50	2.68	0.43	1.89	0.30	1.38	1.44	1.08	1.12
30	9.92	1.09	3.10	0.34	3.76	0.76	1.40	0.28	1.41	1.58	0.92	1.03

One Unit of Peroxidase Activity = change in OD of 10/min.

One Unit of IAA Oxidase Activity = 1 mg IAA destroyed/25 min at 37°C.

Table 45 : Changes in PAL and TAL Activities in Crotalaria Callus Cultures at Different Cycloheximide Concentrations.

Inoculum : 300± 30 mg tissue by fresh weight on 30 ml MS medium supplemented with 10.0, 25.0 and 50.0 mg/l cycloheximide in addition to 2.0 mg/l 2,4-D.

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

Time (day)	10.0				25.0				50.0			
	PAL		TAL		PAL		TAL		PAL		TAL	
	units/ cult.	units/ mg.prot.	units/ cult.	units/ mg.prot.	units/ cult.	units/ mg prot.	units/ cult.	units/ mg prot.	units/ cult.	units/ mg prot.	units/ cult.	units/ mg prot.
0	0.27	0.095	0.067	0.024	0.27	0.095	0.067	0.024	0.027	0.045	0.067	0.024
5	0.38	1.444	0.17	0.640	0.21	1.004	0.12	0.570	0.18	0.409	0.10	0.227
10	3.83	1.885	1.83	0.900	1.98	1.200	1.44	0.870	0.98	1.866	0.40	0.762
15	12.56	1.933	4.84	0.740	10.32	2.059	3.96	0.792	2.25	2.848	1.41	1.78
20	2.35	0.319	0.83	0.110	1.86	0.298	0.71	0.114	1.05	1.091	0.42	0.436
30	1.56	0.172	0.57	0.063	1.02	0.208	0.36	0.073	0.73	0.816	0.28	0.313

One Unit of PAL Activity = formation of 10 umoles of cinnamate/hr at 37°C.
One Unit of TAL Activity = formation of 10 umoles of p-coumarate/hr at 37°C.

Fig. D-49 to D-54. Progressive changes in the development of total (Fig. D-49) and specific (Fig. D-50) activity of IAA oxidase, total (Fig. D-51) and specific (Fig. D-52) activity of PAL and total (Fig. D-53) and specific (Fig. D-54) activity of TAL at various cycloheximide concentrations.

Treatments : MS medium supplemented with 10, 25 or 50 mg/l cycloheximide in addition to 2.0 mg/l 2,4-D.

-□- 10 mg/l cycloheximide,

-O- 25 mg/l cycloheximide, and

-Δ- 50 mg/l cycloheximide.

Experimental details as given in Table 44 and Table 45.

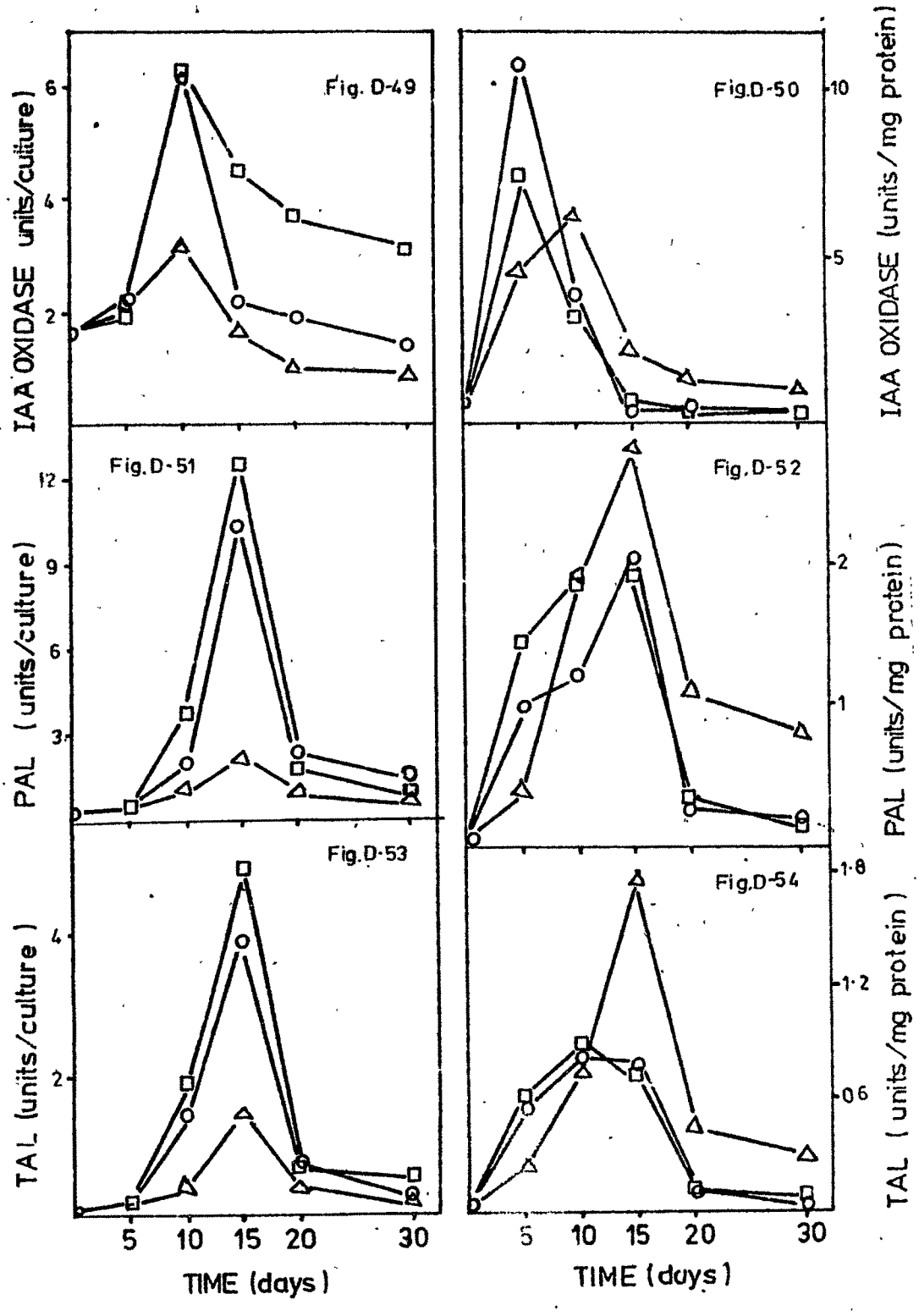


Fig. D-49
Fig.D-50
Fig.D-51
Fig.D-52
Fig.D-53
Fig.D-54
IAA OXIDASE units/culture)
IAA OXIDASE (units/mg protein)
PAL (units/culture)
PAL (units/mg protein)
TAL (units/culture)
TAL (units/mg protein)
TIME (days)
TIME (days)

Fig. D-15 and Table 24), except at 25.0 mg/l cycloheximide level where it registered lower value than the control on day 20. The decline set in on day 5 reached the lowest on day 15 at 10.0 mg/l and on day 20 at 25.0 and 50.0 mg/l cycloheximide concentrations, after which slight recovery in specific activity was recorded.

Unlike peroxidase, no suppression in IAA oxidase activity was observed on day 5(Fig. D-49, Table 44) and it reached a peak on day 10 at all the cycloheximide concentrations. The highest inhibition recorded over the control (maximum 69.13 units/culture on control medium) was 95% at high cycloheximide level. The pattern of specific activity (Fig. D-50) was quite similar to that of the pattern of peroxidase specific activity except that the recovery recorded in the specific activity of peroxidase after the decline was absent in case of IAA oxidase. Also, at 10.0 and 25.0 mg/l cycloheximide, the level of IAA oxidase dropped below the level of control (control supported minimum 0.56 units/mg protein) in contrast to peroxidase activity where it remained higher than the control.

The developmental pattern of PAL (Fig. D-51) and TAL (Fig. D-53) (Table 45) at various cycloheximide

concentrations closely resembled with that of control medium (Figs. D-17 and D-18). However, 54.24% inhibition of PAL and 36.48% inhibition of TAL at low level of cycloheximide and 91.80% of PAL and 81.49% of TAL inhibition at high level of cycloheximide were recorded over the control (highest 27.45 units/culture for PAL and 7.62 units/culture for TAL activities were recorded in control medium).

As was the case with peroxidase and IAA oxidase, initial induction in specific activity of PAL and TAL was observed at all the cycloheximide levels (Figs. D-52 and D-54, Table 45) over the control (maximum 0.68 units/mg protein for PAL and 0.189 units/mg protein for TAL specific activities were registered in control medium). However, the induction period was longer than that of peroxidase and IAA oxidase. In case of PAL, peak in specific activity at all the cycloheximide levels was recorded on day 15, whereas in case of TAL, the peak of the specific activity was attained on day 10 at 10.0 and 25.0 mg/l cycloheximide and on day 15 at 50.0 mg/l cycloheximide concentration.

7. Changes in p-Coumaryl CoA : ligase, Phenylalanine Transaminase and Tyrosine Transaminase during the Course of Growth Cycle.

To examine the changes in ligase and transaminases activities, 300± 30 mg by fresh weight callus pieces were inoculated on 30 ml MS medium supplemented with 2.0 mg/l 2,4-D (control medium). The flasks were incubated in continuous light at 26± 2°C for 30 days. A fixed number of replicate flasks was harvested for assaying the activity of p-coumaryl CoA:ligase, phenylalanine transaminase and tyrosine transaminase as described in Chapter II, Materials and Methods, 7D and 7E.

Fig. D-55 and Table 46 illustrate the progressive changes in p-coumaryl CoA:ligase activity during the culture period of 30 days. Development of the activity was absent for initial 10 days. Increase in the activity set in on day 10 showed maximum development after day 15 till the end of culture period. Thus, like PAL and TAL, p-coumaryl CoA:ligase activity was low during early culture period; but unlike PAL and TAL, the activity showed no decline after its initiation.

Specific activity pattern of p-coumaryl CoA:ligase activity (Fig. D-56) showed a marked suppression for initial 10 days. 10 - 30 days period constituted rapid increase period of the enzyme activity, the increase being

Table 46 : Progressive Changes in p-Coumaryl CoA : ligase During the Culture Period of 30 days.

Inoculum : 300± 30 mg tissue by fresh weight on 30 ml MS medium supplemented with 2.0 mg/l 2,4-D and 2% sucrose.

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

Time (days)	p-Coumaryl CoA : ligase	
	Units/culture	Units/mg protein
0	0.85	0.30
5	0.52	0.06
10	0.96	0.05
15	3.98	0.10
20	12.67	0.22
30	18.52	0.29

One Unit of p-Coumaryl CoA : ligase Activity = formation of 1 umole of cinnamyl CoA/30 min at 37°C.

Table 47 : Progressive Changes in Phenylalanine Transaminase and Tyrosine Transaminase Activities During the Culture Period of 30 Days.

Inoculum : 300± 30 mg tissue by fresh weight on 30 ml MS medium supplemented with 2.0 mg/l 2,4-D and 2% sucrose.

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

Time (days)	Phenylalanine Transaminase		Tyrosine Transaminase	
	units/culture	units/mg prot.	units/culture	units/mg prot.
0	0.9	0.32	1.01	0.35
5	6.19	0.74	5.38	0.64
10	15.64	0.86	13.58	0.75
15	23.20	0.58	26.97	0.67
20	21.20	0.37	24.96	0.43
30	19.34	0.30	20.36	0.32

One Unit of TAT Activity = formation of 100 umoles of phenylpyruvic acid/hr at 37°C.

One Unit of TAT Activity = formation of 100 umoles of hydroxyphenylpyruvic acid/hr at 37°C.

Fig. D-55 and D-56. Progressive changes in the development of total (Fig. D-55) and specific (Fig. D-56) activity of p-Coumaryl:CoA Ligase in the tissues grown on defined medium.

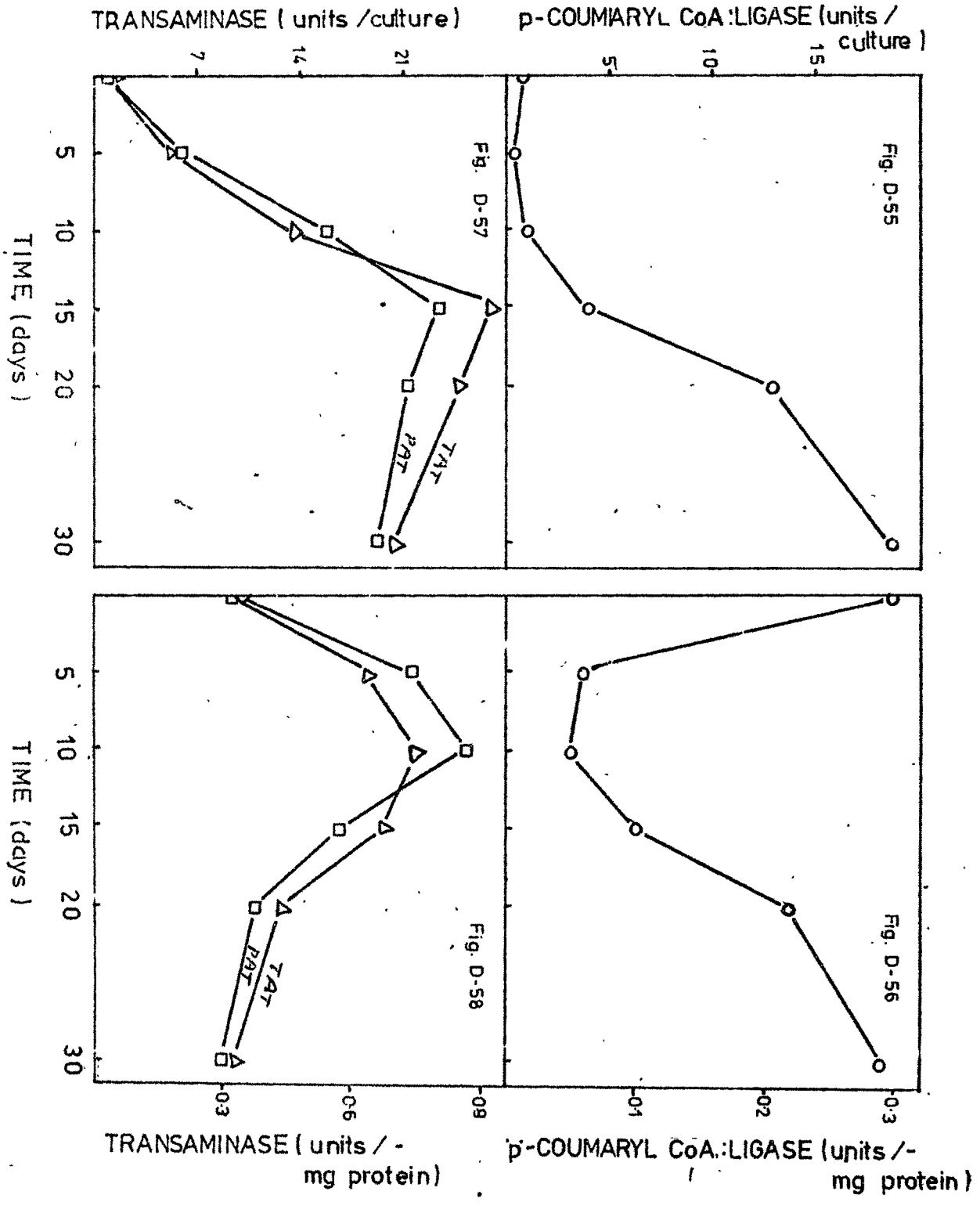
Treatment : MS medium supplemented with 2.0 mg/l 2,4-D (control medium).

Experimental details as given in Table 46.

Fig. D-57 and D-58. Progressive changes in the development of total (Fig. D-57) and specific (Fig. D-58) activities of phenylalanine and tyrosine transaminases in the tissues grown on defined medium.

Treatment : MS medium supplemented with 2.0 mg/l 2,4-D (control medium).

Experimental details as given in Table 47.


TRANSAMINASE (units /culture)
p-COUMARYL CoA:LIGASE (units / culture)
TIME (days)
TRANSAMINASE (units / - mg protein)
p-COUMARYL CoA:LIGASE (units /- mg protein)
TAT
PAT
Fig. D-55
Fig. D-56
Fig. D-57
Fig. D-58

more pronounced from 10 to 20 days. Thus, in contrast to PAL and TAL specific activity patterns where the termination was recorded on day 15, p-coumaryl CoA:ligase specific activity pattern showed no decline after its initiation.

Phenylalanine transaminase and tyrosine transaminase activities are presented in Table 47 and illustrated in Fig. D-57. Nearly linear increase in both the enzymes was registered during 0 - 15 days culture period after which slight fall was noticed. During the early culture period (upto day 10) the values recorded for tyrosine transaminase activity were less than that of phenylalanine transaminase, however, tyrosine transaminase maintained higher activity in the subsequent period. Specific activity of both the enzymes showed the peaks on day 10 (Fig. D-58). As was the case with total transaminases activities, specific activity of tyrosine transaminase maintained higher level than phenylalanine transaminase during the late culture period (15 - 30 days).

SUMMARY

The polyphenol content increased significantly during the period of rapid growth at all levels of all the

substances tested. Increased production of phenolic compounds with increase in sucrose concentration clearly indicated that the synthesis of phenolic materials depended on the availability of carbohydrate during the course of growth cycle. 2,4-D and kinetin affected the initiation of polyphenol synthesis in that the initiation was delayed at higher levels, while GA_3 and NAA showed no such adverse influence on the initiation. Further, growth was more markedly influenced than polyphenol synthesis in the tissues grown on NAA medium. GA_3 supported maximum polyphenol content both on total and mean amount basis. Cycloheximide inhibited growth and polyphenol synthesis at all the levels tested, more inhibition being recorded at higher level.

Light induced synthesis of phenolic compounds. However, at higher GA_3 level more phenolic production was recorded in complete darkness, suggesting the substitution of light stimulation by higher GA_3 level. On the other hand, light inhibited growth in presence of all the hormones tested except at higher GA_3 level. Thus, unlike polyphenol synthesis, reversal of light inhibition of growth at low GA_3 level was observed in presence of higher GA_3 dose.

Increased level of sucrose supported more activity of all the enzymes studied. The developmental pattern of peroxidase, IAA oxidase, PAL and TAL activities corresponded with that of accumulation of phenolic compounds either throughout the culture period or during the part of it. Peroxidase showed close correlation with phenolic accumulation at all the cultural parameters tested, suggesting an important role of peroxidase in polyphenol synthesis. Though PAL and TAL showed relation with the synthesis of phenolic materials only during the part of culture period, the steep increase in their activities at the time of maximum rate of phenolic synthesis proved their undoubtable key roles. IAA oxidase showed both discripancy and similarity with the accumulation of phenolic compounds and its role in the phenolic biosynthesis can be traced only with the role of peroxidase, which is discussed later. Usual inhibition of all the enzymes by cycloheximide was observed. However, specific activity of all the enzymes studied showed increase over the control, suggesting that cycloheximide affected structural proteins more severely than the enzymic proteins.

Light induction of all the enzymes was recorded, the induction being less marked at higher level of hormone

tested. Further, peroxidase at higher level of 2,4-D and GA_3, IAA oxidase at higher level of all the hormones tested and PAL and TAL at higher level of GA_3 showed the reversal of light induction.

Maximum increase in p-coumaryl CoA:ligase activity was recorded after the fall in PAL and TAL activities. Phenylalanine and tyrosine transaminase showed higher activity during initial 15 days. Specific activity of both the enzymes registered a peak on day 10. This suggested that during the early culture period phenylalanine and tyrosine, which are well established precursors of phenyl-propanoid compounds, are diverted more to the primary metabolism rather than used in the production of secondary metabolites.

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E. *IN VIVO* AND *IN VITRO* L-PHENYLALANINE AMMONIA-LYASE FROM *CROTALARIA JUNCEA* L. : PURIFICATION, SUBUNIT STRUCTURE AND KINETIC PROPERTIES.

The aim of the experiments conducted here was to isolate, purify and characterize phenylalanine ammonia-lyase from *Crotalaria* seedlings and callus tissues. More specifically, the aim was to ascertain certain properties of the enzyme such as : substrate specificity, molecular weight, subunit composition and whether the enzyme was regulatory or non-regulatory. Other properties of the enzyme were also investigated during the course of this research which included the optimal requirements for the enzyme assay. Furthermore, the existance of separate tyrosine and phenyl-alanine ammonia-lyases has been postulated to exist in some cases. An initial objective of this research was to ascertain if two such enzymes were present in *Crotalaria*.

PAL has been isolated and purified from three days old *Crotalaria* seedlings and from 15 days old *Crotalaria* callus cultures. Certain properties of the enzyme from both the origins were investigated and the results obtained are incorporated in this section.

1. Purification of Enzyme

Acetone powder was prepared separately from three days old seedling and 15 days old callus of Crotalaria according to the method described in Chapter II, Materials and Methods, 7C. Same purification steps were carried out for the seedling as well as for the callus except for some minor changes. Acetone powder was subjected to the following typical enzyme purification steps which are also summarized in Table 48 (for seedling) and Table 49 (for callus). All the operations were performed at room temperature except where otherwise stipulated.

Step 1. Crude Enzyme : The powder (10 g) was crushed in a precooled mortar along with glass powder and 25 mM Tris-HCl buffer, pH 8.8 (150 ml and 175 ml in case of callus and seedling respectively), passed through a cheese cloth and centrifuged at 10,000 X g for 10 min. The supernatant solution was retained.

Step 2. Treatment with Protamine Sulfate : In case of callus enzyme, one ml of 5% protamine sulfate suspension was added to step 1 supernatant solution for every 200 mg of protein and in case of seedling enzyme one ml of 5%

protamine sulfate suspension was added to step 1 supernatant solution for every 300 mg of protein as measured by the method of Lowry et al. (1951). The protein-protamine sulfate mixture was stirred for 10 min and then centrifuged at 15,000 X g for 10 min. The pellet was discarded.

Step 3. Ammonium Sulfate Fractionation : Solid ammonium sulfate (29.52 g in seedling and 25.26 g in callus supernatant) was added to bring the protein solution to 30% saturation. The mixture was centrifuged after 30 min at 0°C at 10,000 X g for 10 min. The supernatant solution was brought to 65% saturation with (38.52 g in seedling and 32.96 g in callus supernatant) $(NH_4)_2SO_4$. PAL precipitated between 30-65% $(NH_4)_2SO_4$ saturation. The precipitate was dissolved in 25 mM Tris-HCl buffer, pH 8.8 (30 ml) and centrifuged.

Step 4. Acetone Precipitation : The clear supernatant from step 3 was subjected to acetone precipitation at -15°C. Chilled acetone (12.86 ml) was added to the supernatant to bring it to 30% saturation and centrifuged immediately at 10,000 X g for 5 min. Next, the supernatant was brought to 60% acetone saturation and centrifuged for 5 min. The

Table 48 : Summary of Purification of Phenylalanine Ammonia-Lyase from Crotalaria Seedlings *

S T E P	Volume (ml)	Total Protein (mg)	Total Activity (units)	Specific Activity (units/mg protein)	Recovery (%)	Purification (fold)	PAL/TAL Ratio
1. Crude Extract from Acetone Powder (10 g)	175	2310	5775	2.5	100	1.0	1.65
2. Treatment with Protamine Sulfate	180	546	5683	10.4	98.4	4.2	1.92
3. 30 - 65% $(NH_4)_2SO_4$ Fractionation	45	130	4625	35.6	80.0	14.2	1.84
4. 40 - 60% Acetone Precipitation	10	55	4335	78.8	75.1	31.5	1.71
5. D E A E -Cellulose Active Fractions	30	2.16	1164	538.9	20.2	215.6	2.10

* Details of the purification steps are given in the text. One unit is the amount of enzyme that catalyzes the formation of 10 umoles of cinnamate or p-coumarate/hr at 37°C.

Table 49 : Summary of Purification of Phenylalanine Ammonia-Lyase from Crotalaria Callus Cultures*

S T E P	Volume (ml)	Total Protein (mg)	Total Activity (units)	Specific Activity (units/mg protein)	Recovery (%)	Purification (fold)	PAL/TAL Ratio
1. Crude Extract from Acetone Powder (10 g)	150	1255	954	0.76	100	1.0	3.75
2. Treatment with Protamine Sulfate	154	392	909	2.32	95.3	3.1	4.12
3. 30 - 65% $(NH_4)_2$ SO_4 Fractionation	45	80	746	9.33	78.2	12.3	3.91
4. 40 - 60% Acetone Precipitation	10	32.5	692	21.29	75.5	28.0	3.80
5. D E A E -Cellulose Active Fractions	30	1.11	162	145.95	16.98	192.0	4.36

* Details of the purification steps are given in the text. One unit is the amount of enzyme that catalyzes the formation of 10 umoles of cinnamate or p-coumarate/hr at 37°C.

precipitate obtained with 30-60% (v/v) acetone was dissolved in 25 mM Tris-HCl buffer, pH 8.8 (10 ml) and centrifuged. Acetone precipitation step was carried out as fast as possible and traces of acetone were removed from the precipitate under vacuum before dissolving in buffer.

Step 5. DEAE-Cellulose Chromatography : The clear supernatant of step 4 was loaded on a column (15 cm X 1 cm) of DEAE-cellulose pre-equilibrated with Tris-HCl buffer, pH 8.8. The enzyme was eluted with a linear gradient of 75 ml of 25 mM Tris-HCl buffer, pH 8.8, and 75 ml of the same buffer containing 300 mM NaCl. The elution profile of DEAE-cellulose column chromatography is given in Fig. E-1. Two peaks were observed in seedling PAL. However, when fractions from the major peak were collected, pooled and ran through a DEAE-cellulose column again, a similar pattern was recorded. This suggests that aggregation of enzyme molecules was occurring giving rise to a minor peak of PAL. No such minor peak was observed in callus PAL DEAE-cellulose pattern. In both the cases, the active fractions (18-23) were pooled, desalted against glass-distilled water on a column (40 cm X 2 cm) of sephadex G-50 and freeze-dried.

The final preparation moved as a single band on

Fig. E-1. DEAE-cellulose chromatography of L-phenylalanine ammonia-lyase from Crotalaria seedling (I) and callus (II).

Enzyme (10 ml; 4335 (seedling) and 692 (callus) units; 55 (seedling) and 32.5 (callus) mg protein) after the acetone precipitation step was applied to a column (15 cm x 1 cm) of DEAE-cellulose equilibrated with 25 mM Tris-HCl buffer, pH 8.8. The enzyme was eluted with a linear gradient of 0-300 mM NaCl in the above buffer (75 ml of each). Fractions (5 ml) were collected and protein (E_{280}-●-) and PAL (-O-) were measured for each fraction. PAL activity was expressed as E_{278} by incubating 0.1 ml in the case of seedling and 0.2 ml in the case of callus of each fraction for 60 minutes under standard assay conditions. -- - , NaCl conc.

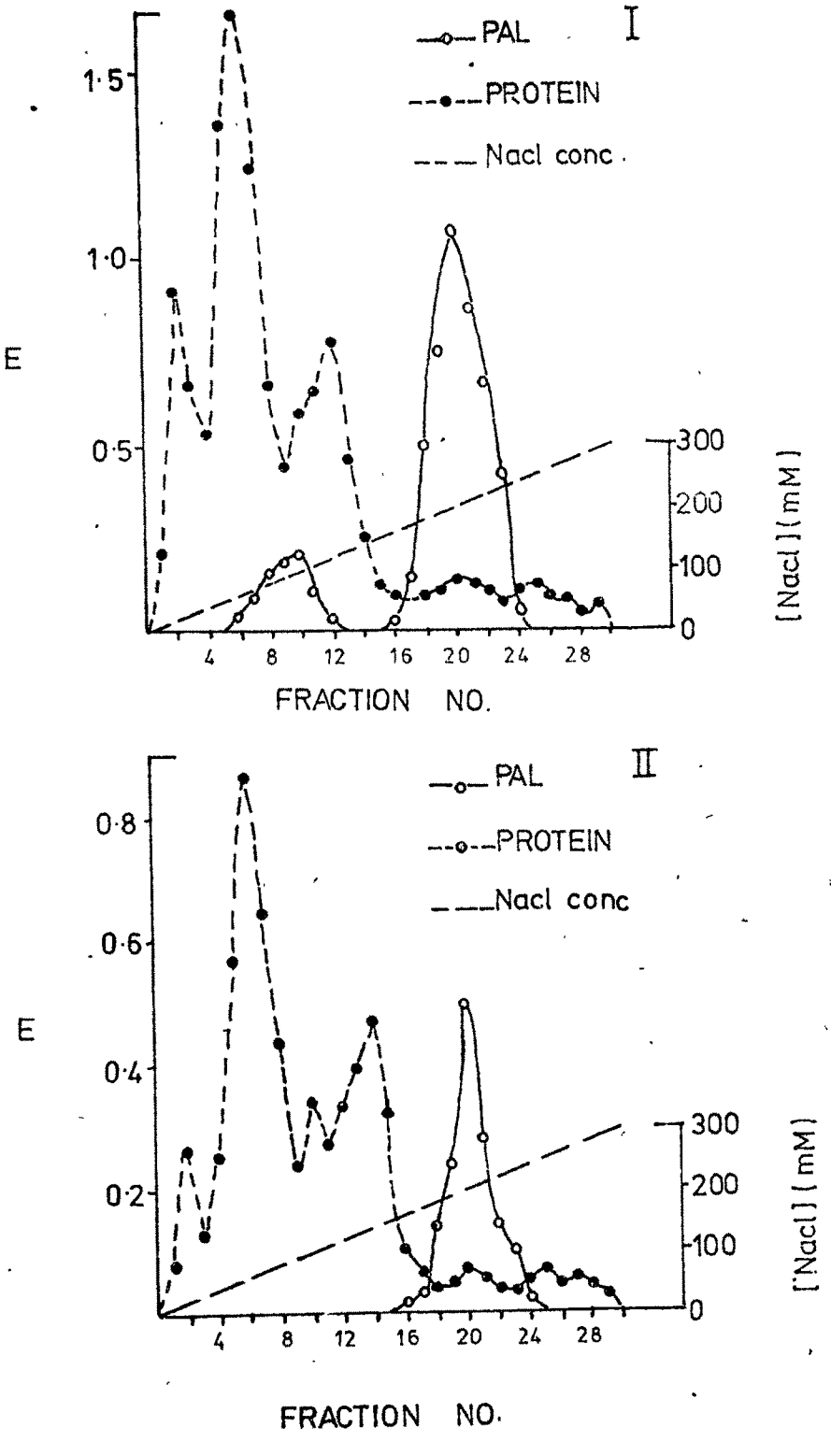

I
PAL
PROTEIN
Nacl conc
E
1·5
1·0
0·5
300
200
100
0
[Nacl] (mM)
4
8
12
16
20
24
28
FRACTION NO.
II
PAL
PROTEIN
Nacl conc
E
0·8
0·6
0·4
0·2
300
200
100
0
[Nacl] (mM)
4
8
12
16
20
24
28
FRACTION NO.

Fig. E-1

polyacrylamide disc gel electrophoresis at pH 8.3. The electrophoresis was performed using 7.5% crosslinked polyacrylamide gels as described in Chapter II, Materials and Methods, 8.

2. General Properties

As summarized in Tables 48 and 49, phenylalanine ammonia-lyase and tyrosine ammonia-lyase activities could not be separated during the purification procedure. The final preparation had the same optimum pH (8.5 - 9.0) and temperature (45 - 50°C) for both the enzymes. The activation energy as determined by Arrhenius plots (Sizer, 1943) for seedling and callus phenylalanine were 32.23 J (7.7 Kcal) and 20.01 J (4.8 Kcal) per mol respectively. PAL retained full activity on heating at 60°C for 1 hr. It was not affected by treatment with metal-chelating agents (8-hydroxyquinoline and EDTA).

3. Substrate Specificity

In addition to L-phenylalanine, the enzyme deaminated L-tyrosine. D-phenylalanine and D-tyrosine did not serve as substrates as tested by the UV spectra of the products.

4. Effect of Carbonyl Reagents

As in the case of PAL from other sources, the Crotalaria enzyme was also sensitive to carbonyl reagents. The effective concentration required to give almost 100% inactivation of the enzyme differed from one reagent to another (Table 50). The reversibility of inhibition by carbonyl reagents was tested by passing the enzyme pretreated with such reagents to cause 50% inactivation or less, through a column (20 cm X 1 cm) of Sephadex G-25 equilibrated with 25 mM Tris-HCl buffer, pH. 8.8, and the elutes were assayed for enzyme activity. The inhibition caused by each of the carbonyl reagents tested was found to be irreversible. Similar observations were made with TAL.

5. Effect of Sulphydryl Reagents

The effect of various sulphydryl reagents on the activity of PAL and TAL were investigated to determine whether PAL and TAL were sulphydryl enzymes. The aliquots of protein fraction were incubated in a range of concentrations of sulphydryl reagents for 10 minutes and the reaction was started by the addition of substrate. PAL and TAL activities are expressed as percentage of respective controls

Table 50 : Effect of Carbonyl Reagents on PAL and TAL Activities from Crotalaria Seedlings.*

Compound	Final Conc. (mM)	Enzyme Activity (%)	
		PAL	TAL
$NaBH_4$	0.005	66	58
	0.01	38	35
	0.02	15	14
	0.10	2	2
NaCN	0.1	92	90
	0.5	36	36
	2.0	0	0
$NaHSO_3$	0.1	98	96
	0.5	46	41
	2.0	0	0
Semicarbazide-HCl	0.1	97	98
	4.0	85	84
	10.0	72	68
Hydroxylamine-HCl	0.1	96	98
	4.0	76	73
	10.0	48	49

* The enzyme was incubated with each of the reagents for 10 min at pH 8.8 and 37°C. The enzyme activities are expressed as percentage of respective controls processed in the absence of carbonyl reagents.

Table 51 : Effect of Sulphydryl Reagents on PAL and TAL Activities from Crotalaria Seedlings. *

Compound	Final Conc. (mM)	Enzyme Activity (%)	
		PAL	TAL
Glutathione	0.5	106	102
	5.0	109	105
Mercaptoethanol	0.5	112	108
	5.0	118	110
Cysteine	0.5	106	103
	5.0	82	86

* The enzyme was incubated with each of the reagents for 10 min at pH 8.8 and 37°C. The enzyme activities are expressed as percentage of respective controls processed in the absence of Sulphydryl reagents.

Table 52 : Effect of Aromatic Compounds on PAL Activity from *Crotalaria* Seedlings and Callus Cultures.*

Compound	Enzyme Activity (%)	
	Seedling	Callus
L-Tyrosine	86	85
D-Tyrosine	32	19
D-Phenylalanine	62	66
P-Hydroxybenzoic acid	99	97
Chlorogenic acid	98	98
Caffeic acid	85	88
Ferulic acid	82	81
Cinnamic acid	71	68
p-Coumaric acid	83	85
Protocatechuic acid	97	95
Coumarin	98	98

* The enzyme was preincubated for 10 min with each compound (0.1 mM) and the reaction was started by the addition of substrate. The enzyme activity is expressed as percentage of control processed in absence of the aromatic compounds.

processed in the absence of sulphydryl reagents.

Table 51 shows that glutathione and mercaptoethanol increased the activity of PAL and TAL at all concentrations tested (106 and 109% PAL and 102 and 105% TAL for glutathione and 112 and 118% PAL and 108 and 110% TAL for mercaptoethanol). Cysteine, on the other hand, inhibited both PAL and TAL activities at higher dose. From the results with glutathione and mercaptoethanol, however, PAL appears to be a sulphydryl enzyme, but reversibility of treatment by iodoacetate or hydroxymercuribenzoate needs to be examined to confirm this fact.

6. Effect of Aromatic Compounds

The effect of a number of aromatic compounds, mostly the metabolites of the shikimic acid pathway and related compounds, are summarized in Table 52. Of the compounds tested only D-phenylalanine, D-tyrosine and trans-cinnamic acid were found significant inhibitors of PAL and TAL activities. Other compounds caused only slight inhibition.

7. Requirements for Enzyme Assay

a. Substrate concentration : The effect of L-phenylalanine

Fig. E-2. Effect of L-phenylalanine concentration on seedling and the callus PAL. The experimental conditions were as described in Chapter II, Materials and Methods, except for the varied concentration of substrate.

Fig. E-3. Effect of the enzyme concentration on the development of PAL (both seedling and the callus). The experimental conditions were as described in Chapter II, Materials and Methods.

Fig. E-4. Time course of seedling and the callus PAL. The experimental conditions as described in Chapter II, Materials and Methods, except for the varied incubation period.

Fig. E-5. Effect of pH on the development of PAL (both seedling and the callus). The experimental conditions as described in Chapter II, Materials and Methods, except for the varied pH value.

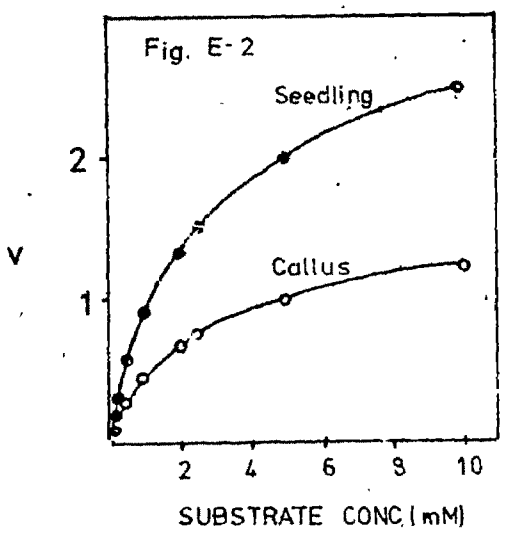


Fig. E-2
Seedling
Callus
V
2
1
2
4
6
8
10
SUBSTRATE CONC.(mM)

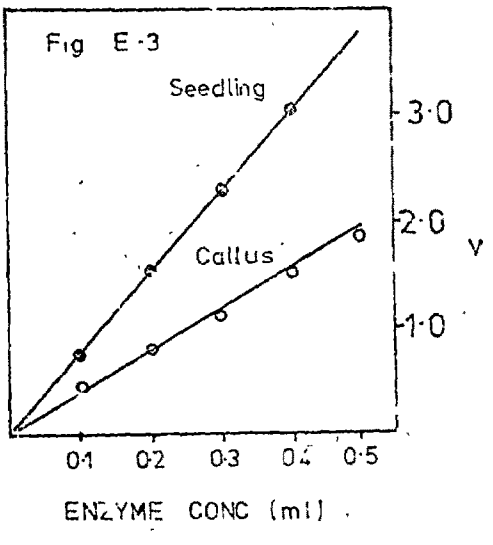


Fig E-3
Seedling
Callus
3·0
2·0
1·0
V
0·1
0·2
0·3
0·4
0·5
ENZYME CONC (ml)

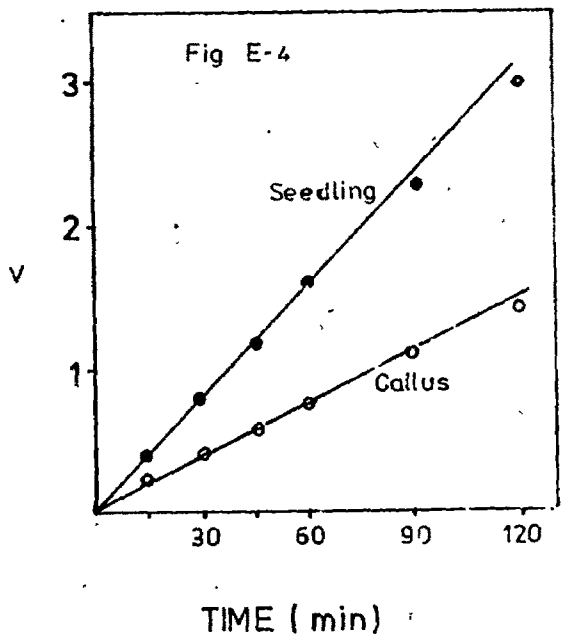


Fig E-4
Seedling
Callus
V
3
2
1
30
60
90
120
TIME (min)

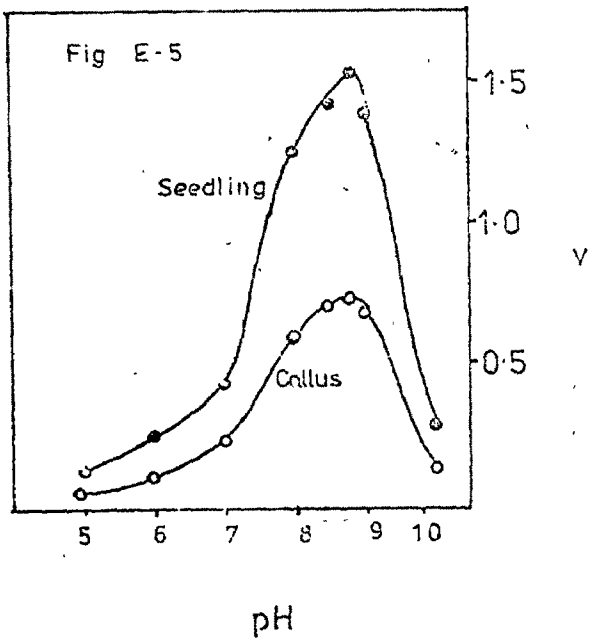


Fig E-5
Seedling
Callus
1·5
1·0
0·5
V
5
6
7
8
9
10
pH

concentration is shown in Fig. E-2. A concentration of 2.5 mM was found to be nearly saturated for both seedling and callus PAL. However, higher concentrations did not inhibit enzyme, but reaction rate was not linear above 2.5 mM L-phenylalanine concentration.

b. Enzyme concentration and Time course of the reaction : The initial velocity in the standard optical assay was proportional to protein concentrations up to more than 1.0 mg protein in case of callus and 1.6 mg protein in case of seedling enzyme (Fig. E-3) (specific activity 1.87 units/mg in callus and 2.36 units/mg in seedling PAL). Similarly, linearity of the reaction was maintained for nearly 90 min with as much as 1.116 units in callus and 2.283 units in seedling enzyme per assay (Fig. E-4).

c. pH : The pH activity of the reaction was determined over the range 5 to 10. The optimal pH of both seedling and callus PAL was found to be 8.5 to 9 (Fig. E-5). PAL from both the origins was also found quite active at pH 8.

d. Temperature and values of activation energy : Reaction mixtures were incubated for 1 hr at 20, 30, 35, 37, 40, 45 and 50^{o}C. The rate of formation of cinnamic acid was linear

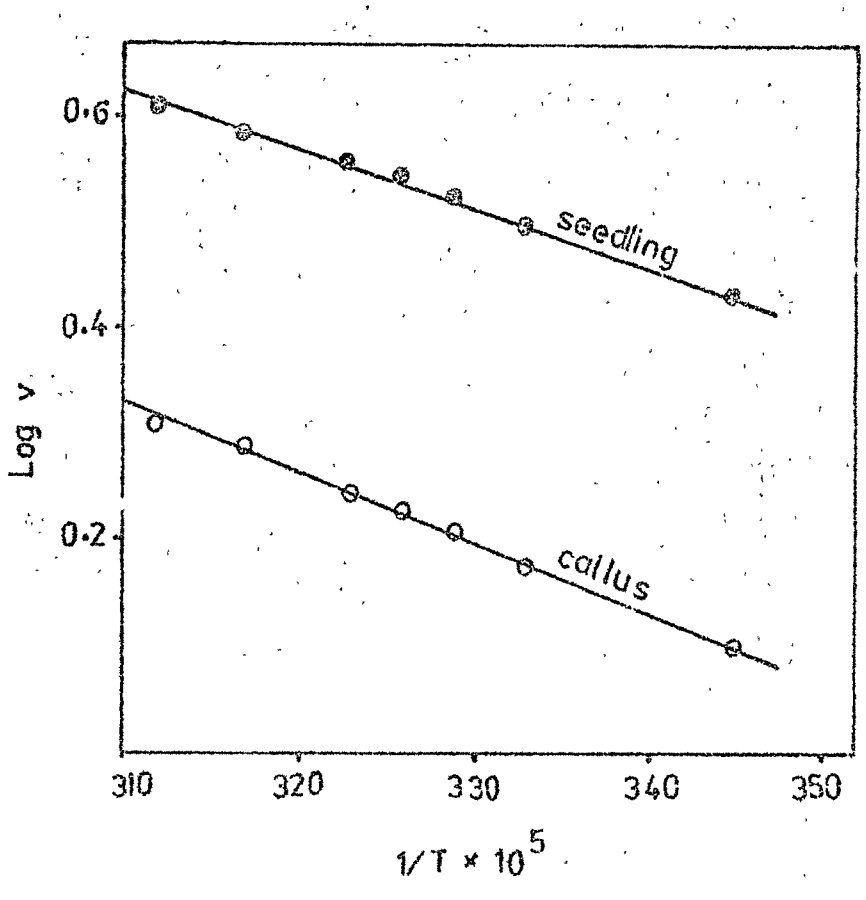


Fig. E-6. Arrhenius plots for PAL from Crotalaria seedling and the callus.

L-phenylalamine ammonia-lyase activity was measured by incubating the purified enzyme at different temperatures for 60 min under standard assay system. The receprocal values of the absolute temperatures(1/T)are plotted against the logarithmic values of initial velocities in the form of Arrhenius plots.

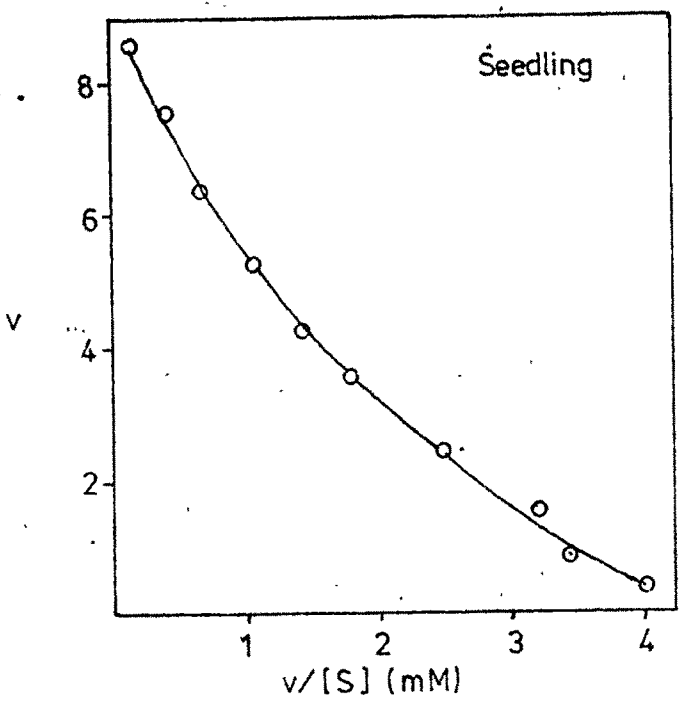
over this range, although the rate of reaction at 50°C deviated somewhat. The logarithms of initial velocities (for 1 hr) were plotted against reciprocal of the absolute temperature (Fig. E-6). The values of activation energy for the deamination of L-phenylalanine in Tris-HCl buffer, pH 8.8, were calculated from the slope of the Arrhenius' plots, and were found to be 7,700 cal. per mol for seedling PAL and 4,800 cal. per mol for callus PAL.

8. Kinetic Analysis of PAL

Studies on initial velocity versus substrate concentration showed a departure from simple Michaelis-Menten kinetics. Substrate-saturation curves indicated that the saturation velocity typical of Michaelis-Menten enzyme was not achieved and the double-reciprocal plots (Lineweaven and Burk, 1934) were biphasic. The same data when plotted as v versus v/(S) (Hofstee, 1959) showed a curvilinear pattern (Fig. E-7) and two Km values could be determined for both seedling and the callus enzyme. Using the nomenclature of Datta and Gest (1965) K_m^H is 4.2 mM and K_m^L is 0.8 mM for seedling PAL and K_m^H is 5.0 mM and K_m^L is 0.4 mM for callus PAL. Thus, apparent Km increased from 0.8 mM to as high as 4.2 mM in

Fig. E-7. Effect of L-phenylalanine concentration on the initial velocity of PAL from Crotalaria seedling and the callus.

L-phenylalanine ammonia-lyase activity was measured by incubating the purified enzyme with different concentrations of L-phenylalanine for 60 min under standard assay conditions. Initial velocities were expressed as units/min and the results are presented in Hofstee plots.



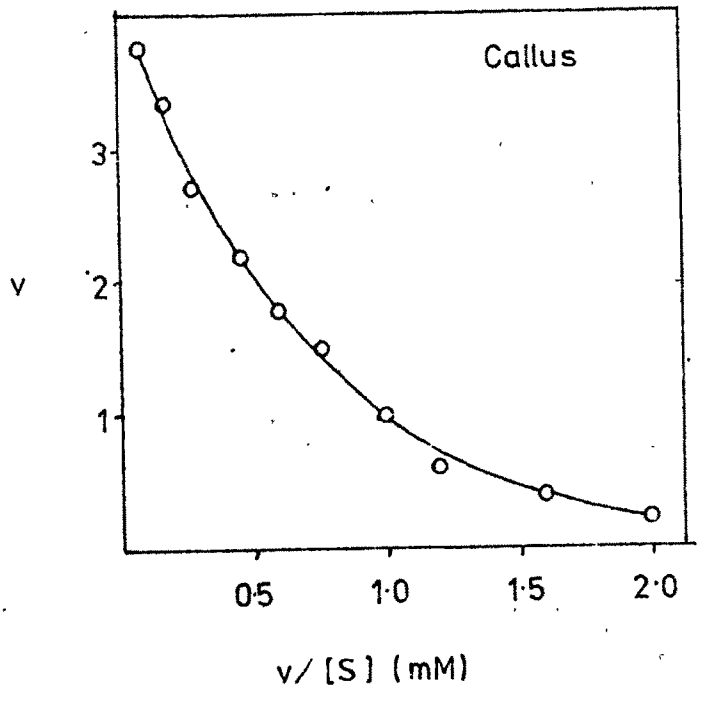


Fig. E-7

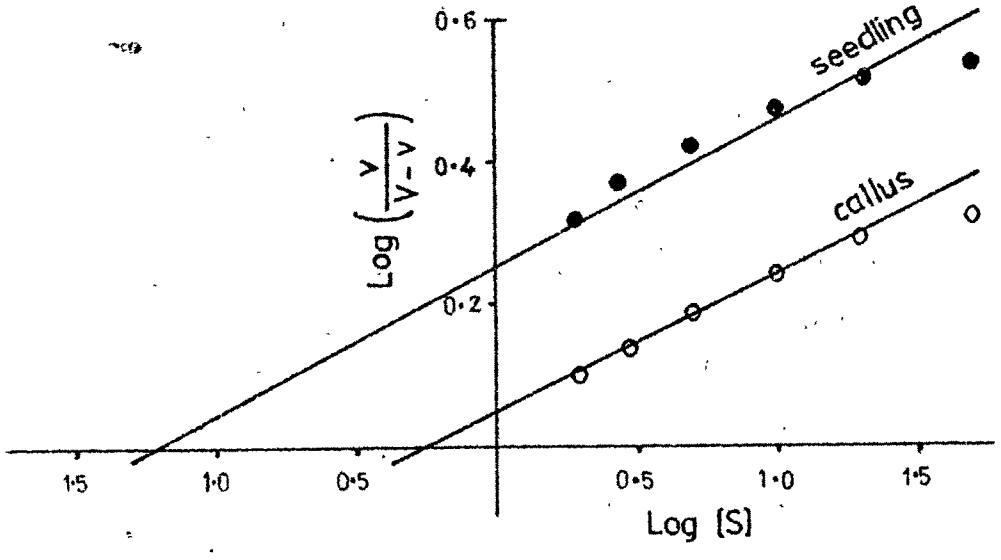


Fig. E-8. Effect of L-phenylalanine concentration on the initial velocity of PAL from Crotalaria seedling and the callus.

L-phenylalanine ammonia-lyase activity was measured by incubating the purified enzyme with different concentrations of L-phenylalanine for 60 min under standard assay conditions. Initial velocities were expressed as units/min and the results are presented in Hill plots.

in case of seedling and 0.4 mM to 5.0 mM in case of callus depending on the substrate concentration and during this process the Vmax increased 7.4-10.0 fold in the seedling and 4.5-6.5 fold in the callus. The value of the Hill coefficient was 0.2, as evident from the Hill plots (Loffield and Eigner, 1969; Atkinson, 1966) constructed from the above kinetic data (Fig. E-8). The Rs values were approximately 86.00 for the seedling enzyme and 88.48 for the callus enzyme.

9. Molecular Weight and Subunit Structure

The molecular weight of the enzyme was estimated on a calibrated Sephadex G-200 column. The standards used for the calibration of the column were : blue dextran (mol. wt. 2,000,000), urease (mol. wt. 483,000), catalase (mol. wt. 244,000), bovine serum albumin (mol. wt. 68,000) and ovalbumin (mol. wt. 43,000). The procedure is described in Chapter II, Materials and Methods, 9. A value of approximately 326,000 was determined for both seedling PAL and callus PAL. This corresponds very closely to the value reported for PAL from other sources.

The method of Weber and Osborn (1969) with 7.5% (w/v)

gels was followed for polyacrylamide gel electrophoresis of proteins in sodium dodecyl sulphate as described in Chapter II, Materials and Methods, 10. Electrophoresis of the enzyme under denaturing conditions yielded two distinct bands (α and β). The values obtained after comparison with the standard polypeptides used as the markers for purpose were α=72,000 and β=90,000. Since the molecular weight of the native PAL is about 326,000, the observed values equate with a subunit pattern of $\alpha_2\beta_2$ for the enzyme.

SUMMARY

L-phenylalanine ammonia-lyase was purified to homogeneity from the acetone-dried powders of three days old seedling and 15 days old Crotalaria callus tissues. The purified enzyme showed bisubstrate activity towards L-phenylalanine and L-tyrosine. Investigation with sulphydryl reagents indicated that PAL is a sulphydryl enzyme, however, it needs further confirmation. It is sensitive to carbonyl reagents and the inhibition is not reversed by gel filtration. The molecular weight of the enzyme, as estimated by Sephadex G-200 chromatography, is around 326,000. The enzyme is made up of two pairs of unidentical subunits, with a molecular

weight of 72,000 (α) and 90,000 (β) respectively. Kinetic analysis suggested that PAL exhibited negative homotropic cooperativity. Two Km values are determined, these are K_m^H 4.2 mM and K_m^L 0.8 mM for the seedling PAL and K_m^H 5.0 mM and K_m^L 0.4 mM for the callus enzyme. The value of Hill coefficient is 0.2 for PAL from both the origins. The activation energy values for the deamination of L-phenylalanine in Tris-HCl buffer, pH. 8.8, are 7.700 cal. per mol for the seedling PAL and 4,800 cal. per mol for the callus PAL. Significant inhibition of the enzyme was exerted by D-phenylalanine, D-tyrosine and trans-cinnamic acid.

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