RESULTS

CHAPTER III

RESULTS

The experiments described in the present Chapter were aimed at successful establishment of tissue cultures of Nicotiana tabacum L. var. Anand-2, to facilitate better understanding of the physiological and biochemical changes involved in growth and differentiation of these tissues.

In order to minimise the degree of variability in the present experimental set up, the factors taken into consideration included environmental uniformity, genetic homogenity of the starting material, and the age and size of the experimental materials. Furthermore, to reduce variability amongst treatments, 5 replicates were harvested at random at a time, pooled and analysed.

The experiments described in this chapter were carried out utilizing : (i) the floral buds, (ii) the floral bud callus, (iii) the organ explants of diploid plantlets, (iv) the anthers, (v) the organ explants of haploid plantlets and (vi) the callus tissues of haploid origin of Nicotiana tabacum L. var. Anand-2.

The general layout of the experiments conducted is

listed below :

Section A : Initiation of Diploid and Haploid Callus Cultures of Nicotiana tabacum L. var. Anand-2.

Expt. 1. Initiation of callus tissues of Diploid origin.

Expt. 2. Initiation of Haploid plantlets through anther culture.

Expt. 3. Initiation of callus tissues of Haploid origin.

Section B - I : Growth and Accumulation of Phenolic Compounds in Diploid Callus Cultures of Nicotiana tabacum L. var. Anand-2.

Expt. 4. Influence of Auxins.

Expt. 5. Influence of Cytokinins.

Expt. 6. Influence of Gibberellic acid (GA_3).

Expt. 7. Influence of various sucrose levels in combination with Auxins and Kinetin.

Section B - II. Growth and Accumulation of Phenolic Compounds in Haploid Callus Cultures of Nicotiana tabacum L. var. Anand-2.

Expt. 8. Influence of Auxins.

Expt. 9. Influence of Cytokinins.

Expt.10. Influence of Gibberellic acid (GA_3).

Expt. 11. Influence of various sucrose levels in combination with auxins and kinetin.

Section C - I :Organogenesis in Diploid Callus Cultures of Nicotiana tabacum L.

Expt. 12. Influence of IAA on organogenesis.

Expt. 13. Influence of GA_3 on organogenesis.

Expt. 14. Influence of other phytohormones and Adenine sulfate on differentiation.

Expt. 15. Influence of IAA and Kinetin interactions on organogenesis.

Expt. 16. Influence of added phenolic acids on organogenesis.

Expt. 17. Organogenesis in root, stem and leaf explants of diploid origin.

Section C - II : Organogenesis in Haploid Callus Cultures of Nicotiana tabacum L.

Expt. 18. Influence of IAA on organogenesis.

Expt. 19. Influence of other phytohormones and adenine sulfate on organogenesis.

Expt. 20. Influence of IAA and kinetin interactions on organogenesis.

Expt. 21. Influence of added phenolic acids on organogenesis.

Expt. 22. Organogenesis in root, shoot and leaf explants of haploid tobacco plantlets.

Section D : Physiological Studies with Peroxidase, IAA Oxidase, MDH, PAL, Peroxidase Isoenzymes and Phenolics during Growth of Diploid and Haploid Callus Tissues of Nicotiana tabacum L.

Expt. 23. Studies with floral buds of N. tabacum during callus initiation.

Expt. 24. Studies with floral bud callus of N. tabacum during its growth on standard medium.

Expt. 25. Studies with haploid callus of N. tabacum during its growth on standard medium.

Section E : Physiological Studies with Peroxidase, IAA Oxidase, MDH, PAL, Peroxidase Isoenzymes and Phenolics in Diploid and Haploid Callus Tissues of Nicotiana tabacum cultured on Root differentiating medium.

Expt. 26. Studies with floral bud callus cultured on root inducing medium.

Expt. 27. Studies with floral bud callus cultured on root inducing medium supplemented with low and high levels of sucrose.

Expt. 28. Studies with floral bud callus cultured on root inducing medium supplemented with various phenolic acids.

Expt. 29. Studies with floral bud callus cultured on root inducing medium containing $\frac{1}{2}$ x and 2 x Mn^{++} ion concentration.

Expt. 30. Studies with Haploid callus tissues cultured on root inducing medium.

Expt. 31. Studies with Haploid callus tissues cultured on root inducing medium supplemented with low and high levels of sucrose.

Expt. 32. Studies with Haploid callus tissues cultured on root inducing medium supplemented with various phenolic acids.

Expt. 33. Studies with Haploid callus tissues cultured on root inducing medium containing $\frac{1}{2}$ x and 2 x Mn^{++} ion concentration.

Section F : Physiological Studies with Peroxidase, IAA Oxidase, MDH, PAL, Peroxidase Isoenzymes and Phenolics in Diploid and Haploid Callus Tissues of Nicotiana tabacum cultured on Shoot differentiating medium.

Expt. 34. Studies with floral bud callus cultured on shoot inducing medium.

Expt. 35. Studies with floral bud callus cultured on shoot inducing medium supplemented with low and high levels of sucrose.

Expt. 36. Studies with floral bud callus cultured on shoot inducing medium supplemented with various phenolic acids.

Expt. 37. Studies with floral bud callus cultured on shoot inducing medium containing $\frac{1}{2}$ x and 2 x Mn^{++} ion concentration.

Expt. 38. Studies with Haploid callus tissues cultured on shoot inducing medium.

Expt. 39. Studies with Haploid callus tissues cultured on shoot inducing medium supplemented with low and high levels of sucrose.

Expt. 40. Studies with Haploid callus tissues cultured on shoot inducing medium supplemented with various phenolic acids.

Expt. 41. Studies with Haploid callus tissues cultured on shoot inducing medium containing $\frac{1}{2}$ x and 2 x Mn^{++} ion concentration.

The results obtained through experimentation are presented in Sections as outlined above.

Section A : Initiation of Diploid and Haploid Callus Cultures of *Nicotiana tabacum* L. var. Anand-2.

Expt. 1. Initiation of callus tissues of diploid origin.

To initiate callus tissues of diploid origin, young flower buds (10-12 mm long) were cut into two identical halves longitudinally and cultured aseptically on MS medium supplemented with 2.0 mg/l each of IAA, NAA and KN, and 2 per cent sucrose.

Callus formation was observed from cut ends of the sepals during the third week in culture. The callus tissues thus initiated were subcultured every 30 days onto fresh MS medium containing 2.0 mg/l each of IAA, NAA and KN, and 2 per cent sucrose. The clones of callus tissues thus built up were green, nodular and friable (Fig. 1).

Expt. 2. Initiation of haploid plantlets through anther culture.

Anthers with uninucleate pollen were excised from flower buds (10-12 mm long) of N. tabacum var. Anand-2 and cultured under aseptic conditions on Nitsch (1969) basal medium (NM) (Table 2; Chapter II), supplemented with 15% coconut milk or 0.2 mg/l IAA, singly or in combination.

Pollen embryogenesis was observed in anthers with uninucleate pollen (Fig. 2). This single nucleus divided to give rise to two nuclei. Both nuclei stained darkly and with

Fig. 1. Diploid callus of floral bud origin of N. tabacum growing on MS medium supplemented with 2.0 mg/l each of IAA, NAA and KN, and 2% sucrose.

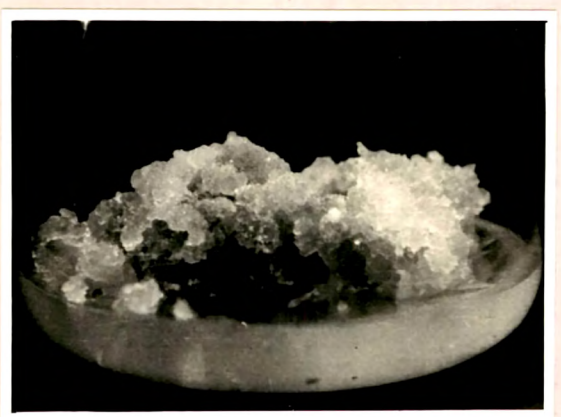

FIG. 1.

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equal intensity (Fig. 2). The two nuclei give rise to multi-celled pollen grain (Fig. 4). The next stage observed was the emerging globular embryo from ruptured pollen wall (Fig. 5). During third week in culture the anthers split open along their lateral walls and young embryoids make their way out (Fig. 6). It is during the fourth week in culture that these embryoids develop into plantlets (Fig. 7). These young plantlets were removed from the anthers and planted on NM with 1% sucrose whereon they developed profuse root system (Fig. 8).

Root tip squashes of these young plantlets demonstrated haploid chromosome number n = 24 (Fig. 9).

Absence of either coconut water or IAA from the NM medium completely suppressed embryoid formation. With the incorporation of either coconut water (15%) or IAA (0.2 mg/l) into the medium only 2 per cent of the cultured anthers supported embryoid formation. However, the combination of IAA (0.2 mg/l) and coconut water (15%) resulted in an enhancement of response to 16 per cent.

Chilling treatment to the anthers, prior to culture, at temperatures ranging from 0-15° for varying lengths of time (2-72 hrs) did not enhance the response.

Pollen Embryogenesis in anthers of N. tabacum on NM with IAA (0.2 mg/l), CM (15%, v/v) and sucrose (2%).

Fig. 2. A pollen grain with a single nucleus and a dead pollen grain.

Fig. 3. Pollen grain with two identical nuclei with equal staining intensity.

Fig. 4. Pollen grain with many cells.

Fig. 5. A globular embryo making its way out of the pollen grain after rupture of the exine.

Fig. 6. A young haploid plantlets emerging out of the lateral walls of the anther.

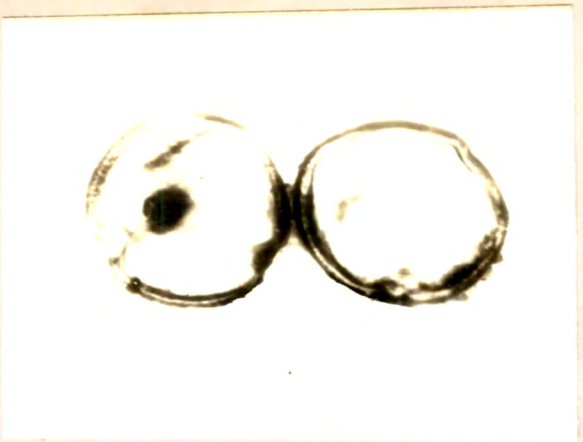


FIG. 2.

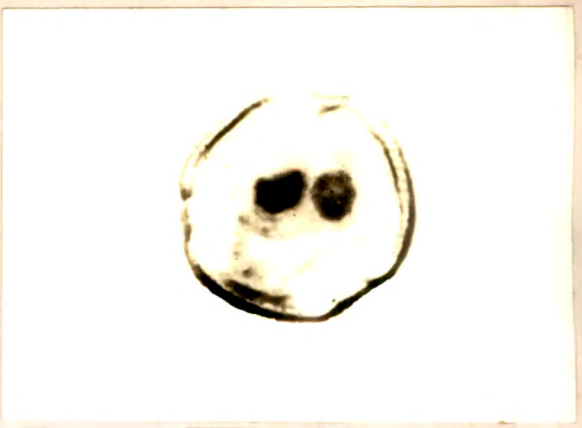


FIG. 3.

FIG. 4.

FIG. 5.

FIG. 6.

Fig. 7. A fully developed young haploid plantlet of *N. tabacum*.

Fig. 8. Haploid plants of *N. tabacum* on NM + 1% sucrose to induce rooting.

Fig. 9. Camera Leucida drawing of chromosomes of haploid *N. tabacum* root tip (n = 24).

Fig. 10. Haploid callus of shoot origin of *N. tabacum* growing on MS medium supplemented with 2.0 mg/l each of IAA, NAA and KN, and 2% sucrose.

FIG. 7.

FIG. 8.

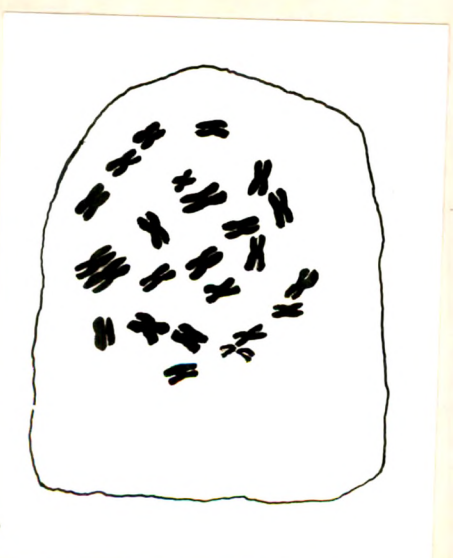


FIG. 9.

FIG. 10.

Expt. 3. Initiation of callus of haploid origin.

The shoots of haploid plantlets were excised and cultured aseptically on the MS basal medium (Table 1; Chapter II) supplemented with 2.0 mg/l each of IAA, NAA and KN, and 2.0 per cent sucrose.

Callus formation was observed all over the shoot explants during the second week in culture. By the end of 4 weeks, callus had proliferated enough to engulf the shoot explant. The callus tissue initiated was lush green, highly granular and friable. By successive transfers every 30 days onto MS medium supplemented with 2.0 mg/l each of IAA, NAA and KN, and 2 per cent sucrose, clones of tissues were built up (Fig. 10).

These callus tissues of haploid and diploid origins were used to study their morphogenetic potentials and growth requirements. Certain key biochemical parameters associated with differentiation were also examined.

Section B-I : Growth and accumulation of Phenolic Compounds in Diploid Callus Cultures of *Nicotiana tabacum* L. var. Anand-2.

B - I. Growth and accumulation of phenolic compounds in diploid callus tissues of N. tabacum L.

To study the hormonal influence on growth and the accumulation of phenolic compounds, callus masses weighing 300±30 mg by fresh weight (12±3 mg dry weight) were incubated on 40 ml agar medium in continuous light at 26±2°. The callus tissues were incubated on MS basal medium for 1 week before hormonal supplements were made to minimize any carry over effects. The tissues were harvested on the completion of 30 days in culture and analysed for fresh and dry weight increases, and also for phenolic accumulation. The results are presented in Tables 5 - 7 and Figures 11 - 20.

Expt. 4. Influence of auxins

Auxins — 2,4-D, IAA, NAA and IBA — were incorporated into the MS basal medium containing 2 per cent sucrose. Each auxin was used at the concentrations of 0.5, 2.0 and 4.0 mg/l in the MS medium to study their effect on growth and phenolic accumulation after 30 days in culture. Callus tissues cultured on MS basal medium acted as the control.

After 30 days of culture the diploid callus tissues increased 2.64 fold in fresh weight and 4.83 fold in dry weight when cultured on MS basal medium. The phenolic

accumulation was 1.15 mg% and 0.66 mg/culture (Fig. 11, Table 5).

With the increase of 2,4-D concentration in the MS medium from 0.5 mg/l through 2.0 mg/l to 4.0 mg/l, the fresh as well as the dry weights increased steadily. On fresh weight basis 0.5, 2.0 and 4.0 mg/l 2,4-D gave 2.44, 2.79 and 3.12 fold increases respectively. Correspondingly dry weight increased 3.73, 3.99 and 4.32 folds respectively. Accumulation of phenolic compounds was 1.77 mg% and 0.795 mg/culture on 0.5 mg/l 2,4-D medium. For 2.0 mg/l 2,4-D medium it was 1.27 mg% and 0.61 mg/culture, and for 4.0 mg/l 2,4-D medium 1.07 mg% and 0.55 mg/culture (Fig. 12, Table 5).

The callus tissues on all the three media was pale yellow in colour and became black with the passage of time in culture.

As in the previous case IAA was incorporated in three different concentrations of 0.5, 2.0 and 4.0 mg/l into the MS basal medium. The fold-wise increases in fresh weight on these three media were 4.86, 4.47 and 4.84 respectively, and the fold-wise dry weight increases were 7.77, 7.45 and 8.8 respectively. Maximum accumulation of phenolic compounds was supported by 0.5 mg/l IAA, the actual values being 2.02 mg%

Fig. 11. Histogram depicting growth (fresh and dry wt.) and polyphenol accumulation in 30 day old cultures of diploid tobacco callus cultured on:

MS basal + 2% sucrose

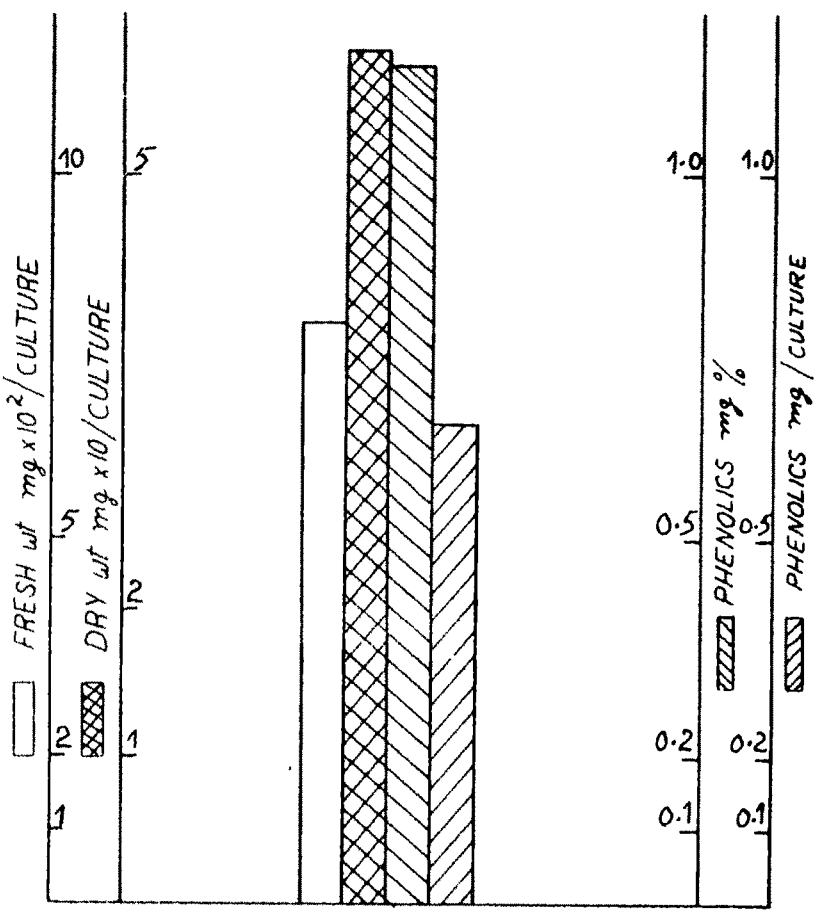

FRESH wt mg x 10²/CULTURE
DRY wt mg x 10/CULTURE
PHENOLICS mg %
PHENOLICS mg/CULTURE
10
5
2
1
5
2
1
1.0
0.5
0.2
0.1
1.0
0.5
0.2
0.1

FIG 11

Fig. 12. Histogram depicting growth (fresh and dry wt.) and polyphenol accumulation in 30 day old cultures of diploid tobacco callus cultured on:

A : MS basal + 2% sucrose.

B : MS + 0.5 mg/l 2,4-D + 2% sucrose

C : MS + 2.0 mg/l 2,4-D + 2% sucrose

D : MS + 4.0 mg/l 2,4-D + 2% sucrose

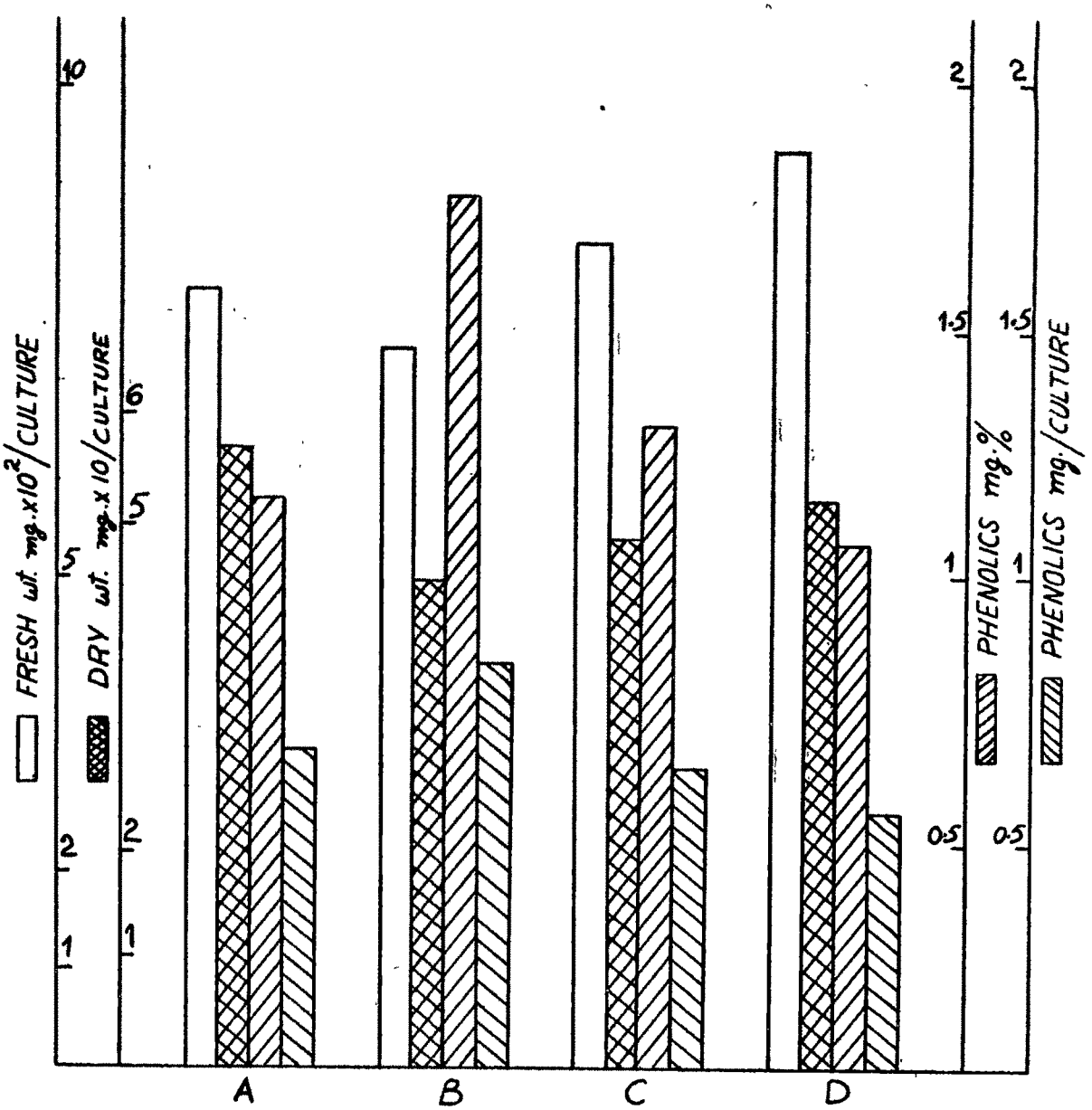

FRESH wt. mg.x10²/CULTURE
DRY wt. mg.x10/CULTURE
PHENOLICS mg.%
PHENOLICS mg./CULTURE
10
5
2
1
6
2
1
2
1.5
1
0.5
2
1.5
1
0.5
A
B
C
D

FIG. 12

and 1.87 mg/culture. The phenolic accumulation decreased with the increase in IAA concentration in the medium. 2.0 mg/l IAA facilitated phenolic accumulation to the tune of 1.77 mg% and 1.59 mg/culture; whereas 4.0 mg/l IAA supported only 1.4 mg% and 1.47 mg/culture of phenolics (Fig. 13, Table 5).

The callus tissues grown on 2.0 mg/l IAA containing medium remained green, nodular and friable. On the other two media though the callus was initially green, it turned brown during the third week in culture.

In order to study the influence of NAA on growth and phenolics, it was incorporated in three concentrations of 0.5, 2.0 and 4.0 mg/l into the MS basal medium. The respective fold-wise increases in fresh weight were 5.75, 8.65 and 4.53 respectively. The corresponding fold-wise dry weight increases were 8.37, 9.81 and 7.01. NAA at 0.5 mg/l gave phenolic accumulation of 1.9 mg% and 1.9 mg/culture; whereas 2.0 and 4.0 mg/l NAA gave 1.65 mg% each and 1.94 and 1.38 mg/culture respectively (Fig. 14, Table 5).

On all the three media the callus tissues were green and soft.

Of the three concentrations of IBA (0.5, 2.0 and 4.0

Fig. 13. Histogram depicting growth (fresh and dry wt.) and polyphenol accumulation in 30 day old cultures of diploid tobacco callus cultured on:

A :MS Basal + 2% sucrose

B :MS + 0.5 mg/l IAA + 2% sucrose

C :MS + 2.0 mg/l IAA + 2% sucrose

D : MS + 4.0 mg/l IAA + 2% sucrose

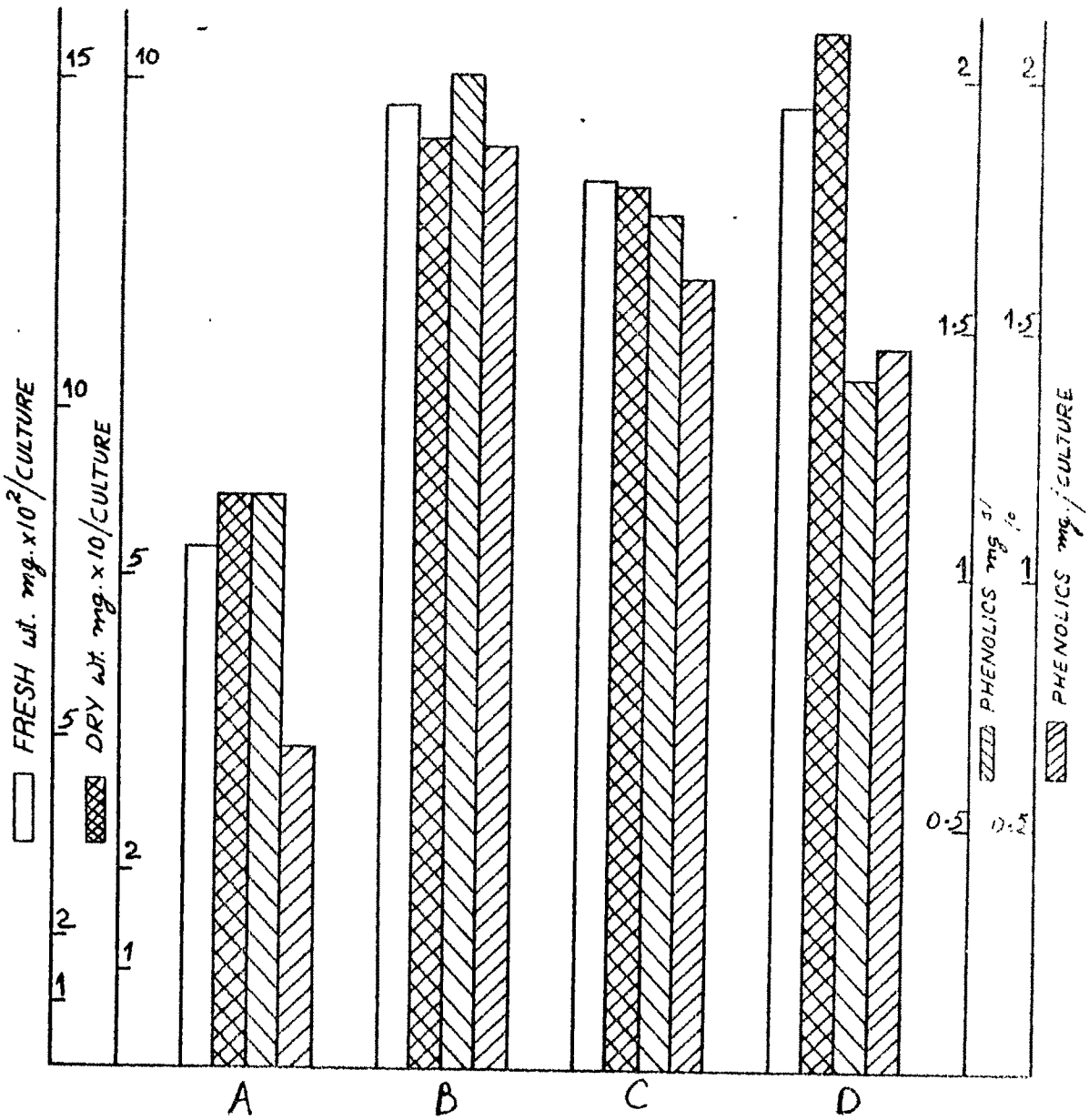

FRESH wt. mg.x10²/CULTURE
DRY wt. mg.x10/CULTURE
PHENOLICS mg %
PHENOLICS mg./CULTURE
15
10
5
2
1
1.5
0.5
A
B
C
D

FIG. 13

Fig. 14 : Histogram depicting growth (fresh and dry wt.) and polyphenol accumulation in 30 day old cultures of diploid tobacco callus cultured on :

A : MS basal + 2% sucrose

B : MS + 0.5 mg/l NAA + 2% sucrose

C : MS + 2.0 mg/l NAA + 2% sucrose

D : MS + 4.0 mg/l NAA + 2% sucrose

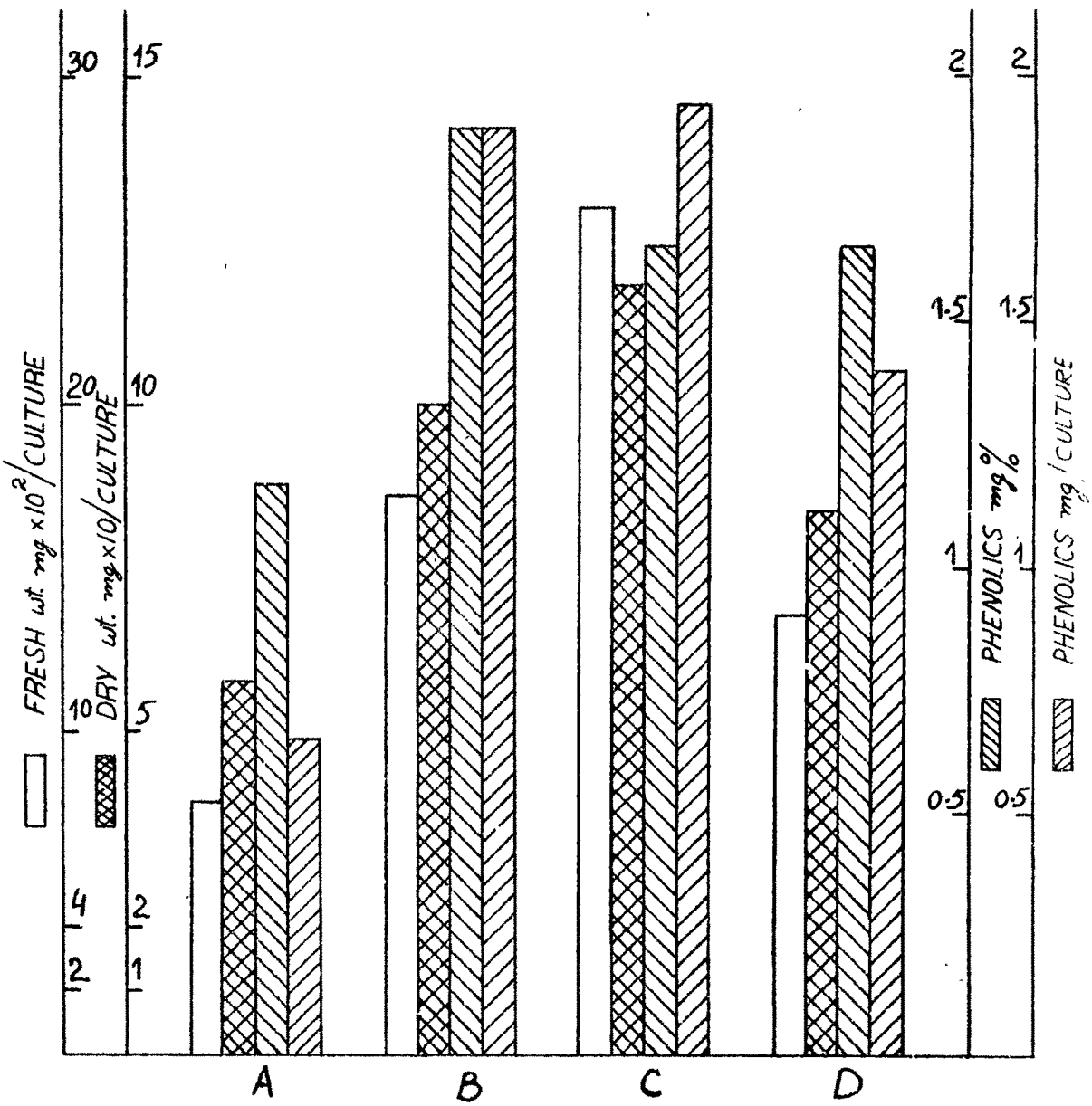

FRESH wt. mg x10²/CULTURE
DRY wt. mg x10/CULTURE
PHENOLICS %
PHENOLICS mg/CULTURE
30
20
10
4
2
15
10
5
2
1
2
1.5
1
0.5
2
1.5
1
0.5
A
B
C
D

FIG. 14

Table : 5. Auxin effect on growth and accumulation of phenolic compounds in diploid callus of Nicotiana tabacum.

Inoculum : 300±30 mg callus tissue by fresh weight (12±3 mg dry weight) on 40 ml MS basal medium with 2% sucrose and various auxins at different concentrations.

Incubation: 30 days in light at 25±2°.

Auxin	Concentration (mg/l)	Fresh weight (mg/culture)	Dry weight (mg/culture)	Phenolics (mg%)	Phenolics (mg/culture)
-	0	794.62 (±21.6)	58.03 (±6.1)	1.15	0.667
2,4-D	0.5	734.04 (±21.5)	44.83 (±5.0)	1.77	0.795
"	2.0	837.22 (±38.1)	47.90 (±5.1)	1.27	0.610
"	4.0	938.03 (±44.3)	51.93 (±8.3)	1.07	0.555
IAA	0.5	1458.72 (±110.6)	93.38 (±4.7)	2.02	1.878
"	2.0	1343.6 (±109.9)	89.59 (±9.9)	1.77	1.590
"	4.0	14.53.6 (± 183.2)	105.61 (±8.7)	1.40	1.484
NAA	0.5	1725.13 (±79.6)	100.51 (±11.6)	1.90	1.909
"	2.0	2595.75 (±201.8)	117.86 (±18.2)	1.65	1.944
"	4.0	1361.25 (±92.9)	84.20 (±13.2)	1.65	1.389
IBA	0.5	1653.47 (±134.7)	106.01 (±10.6)	1.15	1.219
"	2.0	1183.2 (±131.8)	69.17 (±8.8)	1.17	0.812
"	4.0	1288.39 (±139.2)	74.80 (9.3)	1.52	1.140

Data represents an average of 5 replicates.

Figures in parenthesis are standard errors.

mg/l) tested, 0.5 mg/l gave maximum fresh and dry weight increases. The fold-wise values were 5.51 and 8.83 respectively. Next highest values were obtained with 4.0 mg/l IBA. The fresh weight increased 4.29 folds and the dry weight by 6.23 folds. 2.0 mg/l IBA gave 3.94 fold increase in fresh weight and 5.75 fold increase in dry weight. The phenolic accumulation on percentage basis declined with the decrease of IBA concentration in the medium. The three concentrations of IBA - 4.0, 2.0 and 0.5 mg/l - gavephenolic accumulation in the descending order of 1.52, 1.17 and 1.15 mg%. On mg/culture basis maximum accumulation was on 0.5 mg/l IBA followed by 4.0 and 2.0 mg/l IBA respectively. The actual values were 1.21, 1.14 and 0.81 mg/culture respectively (Fig. 15, Table 5).

The callus tissues grown on IBA containing medium were green, nodular and very compact.

Expt. 5. Influence of cytokinins

To study the influence of cytokinins on growth and phenolic accumulation in floral bud callus of N. tabacum the two cytokinins, kinetin and 6-dimethyl-amino purine, were incorporated into MS basal medium containing 2% sucrose. The two cytokinins were used in the concentrations of 0.1, 0.4 and 1.0 mg/l. The callus tissues were grown on the above

Fig. 15. Histogram depicting growth (fresh and dry wt.) and polyphenol accumulation in 30 day old cultures of diploid tobacco callus cultured on :

A : MS basal + 2% sucrose

B : MS + 0.5 mg/l IBA + 2% sucrose

C : MS + 2.0 mg/l IBA + 2% sucrose

D : MS + 4.0 mg/l IBA + 2% sucrose

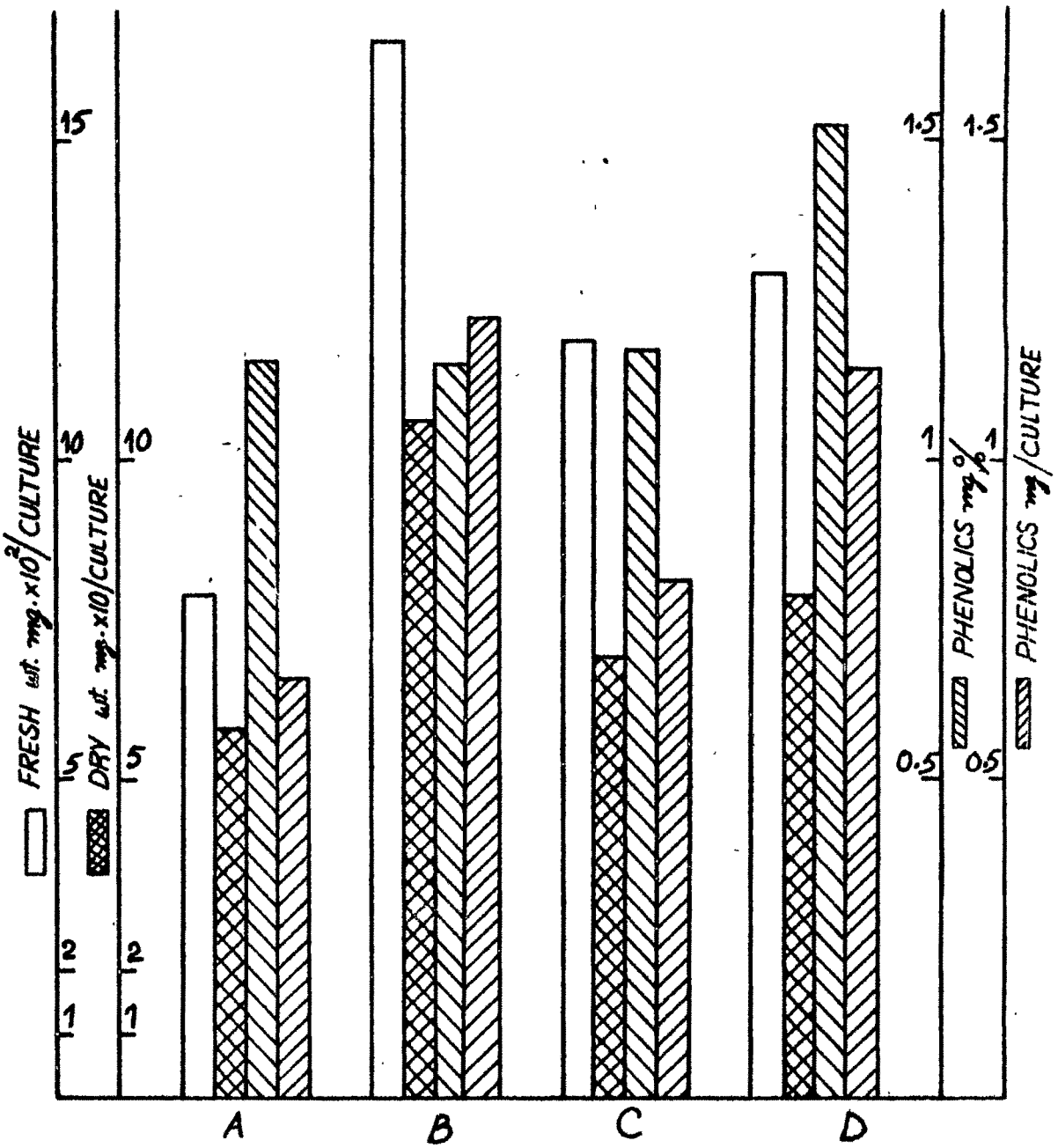

FRESH wt. mg.×10²/CULTURE
DRY wt. mg.×10/CULTURE
PHENOLICS mg%
PHENOLICS mg/CULTURE
15
10
5
2
1
1.5
1
0.5
A
B
C
D

FIG. 15

mentioned media for a period of 30 days before analysis for growth and phenolic accumulation.

On fresh weight basis maximum growth of the callus tissues was obtained on medium containing 0.1 mg/l KN, the fold-wise increase being 4.62 over the inoculum. 3.92 and 4.34 fold increases were registered on media containing 0.4 and 1.0 mg/l KN respectively. However, on the dry weight basis 7.45 fold increase was registered on medium containing 1.0 mg/l KN. 0.1 and 0.4 mg/l KN containing media supported 6.75 and 6.74 fold increases in dry weight. Both on percentage basis and culture-wise, the increase of KN concentration in the medium resulted in decrease in the phenolic accumulation. The actual values were 1.77 mg % and 1.44 mg/culture for 0.1 mg/l KN; 1.65 mg% and 1.33 mg/culture for 0.4 mg/l KN and 1.4 mg% and 1.25 mg/culture for 1.0 mg/l KN respectively (Fig. 16, Table 6).

On all the three media the tissue was deep green, nodular and compact.

The other cytokinin, 6-dimethylamino purine (MAP), did not support growth as well as KN. With the increase of MAP concentration in the medium from 0.1 to 1.0 mg/l the growth declined on both fresh and dry weight basis. The fold-wise increases in fresh weight were 2.55 for 0.1 mg/l MAP,

Table : 6. Cytokinin and Gibberellin effect on growth and accumulation of phenolic compounds in diploid callus of Nicotiana tabacum.

Inoculum : 300±30 mg callus tissue by fresh weight (12±3 mg dry weight) on 40 ml of MS basal medium with 2% sucrose, and various levels of cytokinins and GA_3.

Incubation : 30 days in light at 25±2°.

Phytohormone	Concentration (mg/l)	Fresh weight (mg/culture)	Dry weight (mg/culture)	Phenolics (mg%)	Phenolics (mg/culture)
-	-	794.62 (±21.6)	52.03 (±6.1)	1.15	0.66
KN	0.1	1386.9 (±99.4)	81.19 (±7.4)	1.77	1.44
"	0.4	1177.7 (±78.3)	80.93 (±6.3)	1.65	1.33
"	1.0	1304.3 (±103.7)	89.55 (±3.8)	1.4	1.25
MAP	0.1	766.2 (±65.8)	77.55 (±9.5)	1.32	1.02
"	0.4	525.4 (±25.4)	58.7 (±4.3)	1.16	0.68
"	1.0	493.1 (±43.1)	36.5 (±4.1)	1.65	0.60
GA_3	10.0	678.7 (±36.8)	51.45 (±8.1)	1.84	0.94
"	50.0	917.4 (±74.3)	64.41 (±3.8)	1.52	0.97
"	100.0	1222.5 (±106.6)	87.4 (±8.6)	1.42	1.24

Data represents an average of 5 replicates.

Figures in parenthesis are standard error.

Fig. 16 : Histogram dipicting growth (fresh and dry wt.) and polyphenol accumulation in 30 day old cultures of diploid tobacco callus cultured on :

A : MS basal + 2% sucrose

B : MS + 0.1 mg/l KN + 2% sucrose

C : MS + 0.4 mg/l KN + 2% sucrose

D : MS + 1.0 mg/l KN + 2% sucrose

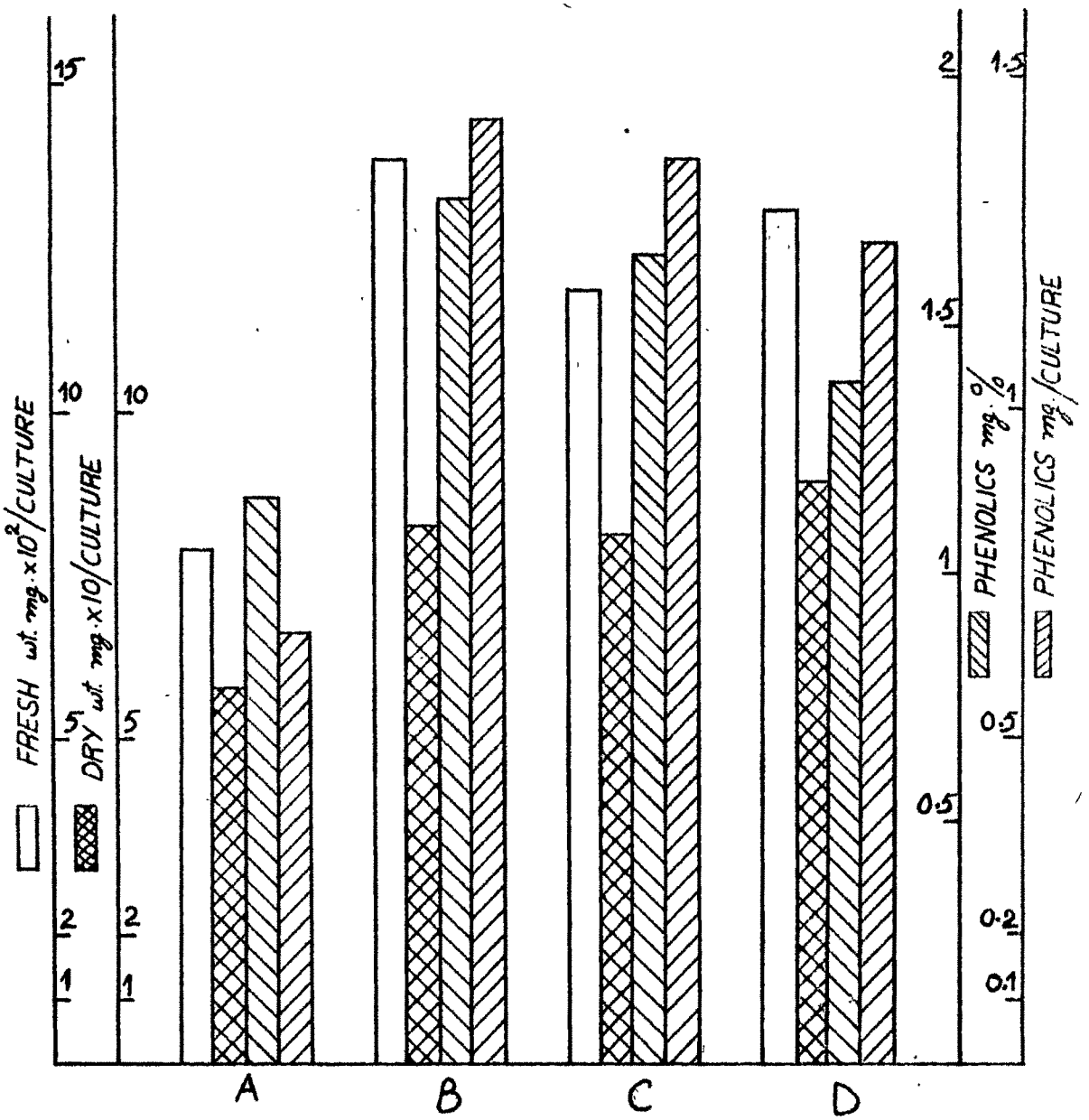

FRESH wt. mg. x 10²/CULTURE
DRY wt. mg. x 10/CULTURE
PHENOLICS mg. %
PHENOLICS mg./CULTURE
15
10
5
2
1
10
5
2
1
2
1.5
1
0.5
1.5
1
0.5
0.2
0.1
A
B
C
D

FIG. 16

1.75 for 0.4 mg/l MAP and 1.64 for 1.0 mg/l MAP respectively. On dry weight basis the respective fold-wise increases were 6.45 for 0.1 mg/l MAP, 4.89 for 0.4 mg/l MAP and 3.04 for 1.0 mg/l MAP. The phenolic accumulation was 1.32 mg% and 1.02 mg/culture for 0.1 mg/l MAP; 1.16 mg% and 0.68 mg/culture for 0.4 mg/l MAP and 1.65 mg% and 0.60 mg/culture for 1.0 mg/l MAP (Fig. 17, Table 6).

In all the cases the callus was dark brown to black and highly compact.

Expt. 6. Influence of Gibberellic Acid (GA_3)

Gibberellic acid (GA_3) was incorporated in concentrations of 10.0, 50.0 and 100.0 mg/l in the MS basal medium to study its effect on growth and phenolic accumulation in diploid callus tissues of N. tabacum. The callus tissues were grown on the above media for a period of 30 days prior to analysis.

GA_3 at 10.0 mg/l concentration supported 2.26 and 4.28 fold increases in fresh and dry weight. At 50.0 mg/l GA_3 the increases were 3.05 and 5.36 fold in fresh and dry weight respectively. 100.0 mg/l GA_3 supported 4.07 and 7.28 fold increases in fresh and dry weights. With the increase of GA_3 concentrations from 10.0 mg/l to 100 mg/l the phenolic accumulation on percentage basis declined. The actual values

Fig. 17. Histogram dipicting growth (fresh and dry wt.) and polyphenol accumulation in 30 day old cultures of diploid tobacco callus cultured on :

A : MS basal + 2% sucrose

B : MS + 0.1 mg/l MAP + 2% sucrose

C : MS + 0.4 mg/l MAP + 2% sucrose

D : MS + 1.0 mg/l MAP + 2% sucrose

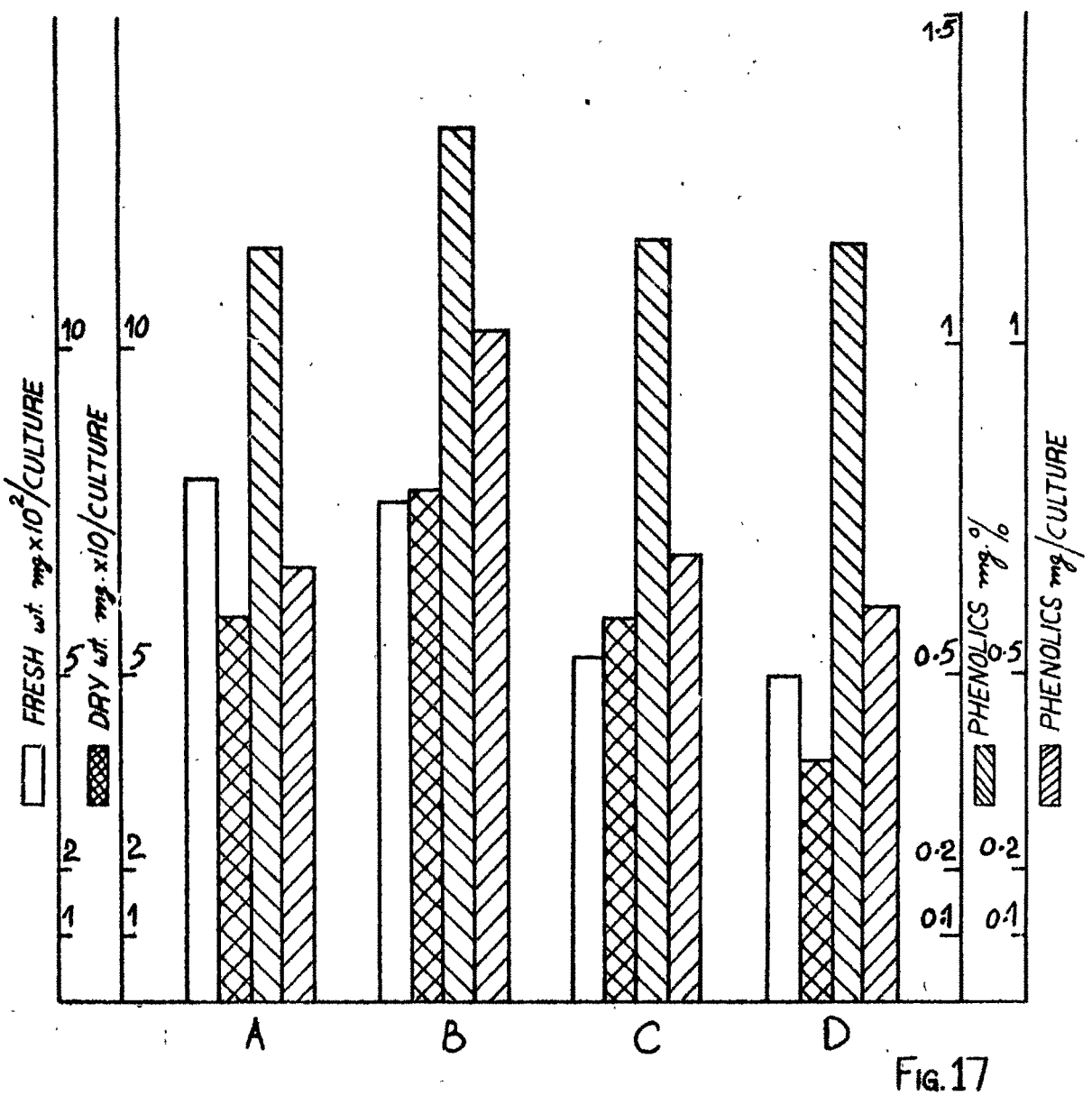

FRESH wt. mg.×10²/CULTURE
DRY wt. mg.×10/CULTURE
PHENOLICS % dw
PHENOLICS mg/CULTURE
10
5
2
1
1·5
1
0·5
0·2
0·1
A
B
C
D

FIG. 17

were 1.84 mg% and 0.94 mg/culture for 10.0 mg/l GA_3, 1.52 mg% and 0.97 mg/culture for 50.0 mg/l GA_3 and 1.42 mg% and 1.24 mg/culture for 100.0 mg/l GA_3 (Fig. 18, Table 6).

All the three concentrations of GA_3 supported callus tissues that were green and friable.

Expt. 7. Influence of various sucrose levels in combination with auxins and kinetin

Auxins and kinetin were taken in combination at different levels of sucrose to study their influence on the growth and phenolic accumulation in diploid callus tissues of N. tabacum. The callus tissues were kept for one week on MS basal medium prior to its inoculation on media containing phytohormones to minimize any carry over effect. The callus tissues were incubated over a period of 30 days before analysis for growth and phenolics.

IAA (2.0 mg/l) and KN (0.4 mg/l) were taken in combination and sucrose added in concentration ranging from 2 to 4%. 13.16 fold increase in fresh weight and 18.02 fold increase in dry weight were gained on IAA (2.0 mg/l) and KN (0.4 mg/l) containing medium supplemented with 2% sucrose. With the increase of sucrose concentration to 3% in the above medium the fresh weight increased 36.34 fold and the dry weight

Fig. 18. Histogram depicting growth (fresh and dry wt.) and polyphenol accumulation in 30 day old cultures of diploid tobacco callus cultured on :

A : MS basal + 2% sucrose

B : MS + 10 mg/l GA_3 + 2% sucrose

C : MS + 50 mg/l GA_3 + 2% sucrose

D : MS +100 mg/l GA_3 + 2% sucrose

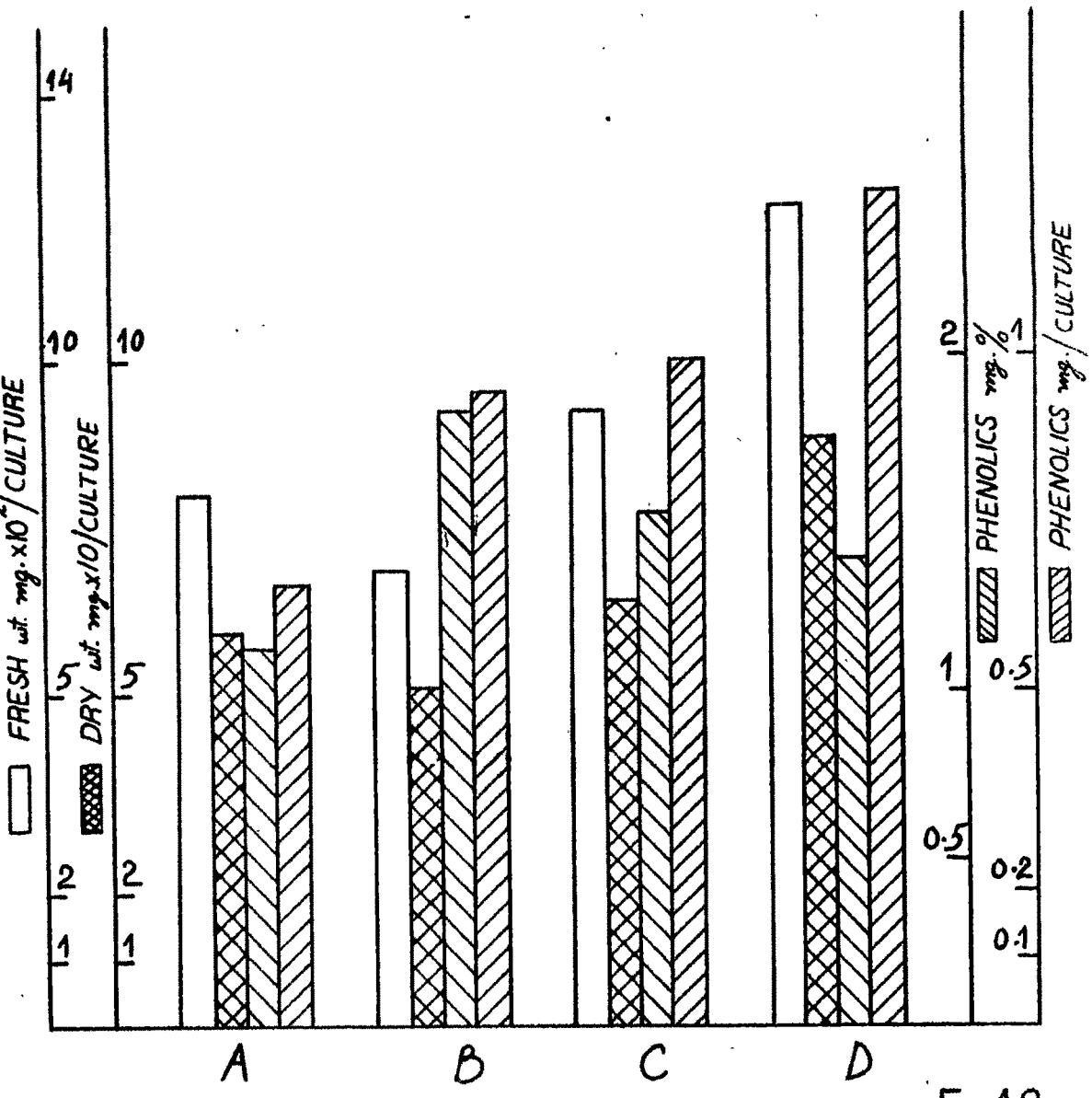

FRESH wt. mg. x10²/CULTURE
DRY wt. mg. x10/CULTURE
PHENOLICS mg. %
PHENOLICS mg./CULTURE
14
10
5
2
1
0.5
0.2
0.1
A
B
C
D

FIG. 18

28.01 fold. The increase of sucrose concentration in the medium to 4% resulted in decline of growth. Sucrose at 4% supported 13.84 and 21.21 fold increases in fresh and dry weight respectively. The phenolic accumulation was 1.12 mg% and 2.61 mg/culture for 2% sucrose medium; 1.84 mg% and 6.18 mg/culture for 3% sucrose medium, and 2.16 mg% and 5.49 mg/culture for 4% sucrose medium (Fig. 19, Table 7).

The callus tissue was green on medium supplemented with 2 and 3% sucrose.The callus tissues, however, turned dark on media supplemented with 4% sucrose. The callus tissue was friable on 3% sucrose medium. On other media, it was nodular and compact.

For yet another experiment auxins, IAA and NAA were taken in combinations with KN. Two combinations tried were 2.0 mg/l IAA + 2.0 mg/l NAA + 0.4 mg/l KN and 2.0 mg/l IAA + 2.0 mg/l NAA + 2.0 mg/l KN. The hormones were supplemented into MS basal medium containing 2% sucrose. The callus tissues were incubated on the above media for 30 days before analysis for growth and phenolic accumulation.

The first of the two media i.e., IAA (2.0 mg/l), NAA (2.0 mg/l) and KN (0.4 mg/l) containing medium gave 54.9 fold increase in fresh weight and 30.74 fold increase in dry weight. When IAA, NAA and KN were used in the concentration of 2.0 mg/l each in

Table : 7. Effect of sucrose level in presence of auxin and kinetin on growth and accumulation of phenolic compounds in diploid callus of Nicotiana tabacum.

Inoculum : 300±30 mg tissue by fresh weight (12±3 mg dry weight) on 40 ml MS medium supplemented with :

A. 2.0 mg/l IAA + 0.4 mg/l KN + 2% sucrose
B. 2.0 mg/l IAA + 0.4 mg/l KN + 3% sucrose
C. 2.0 mg/l IAA + 0.4 mg/l KN + 4% sucrose
D. 2.0 mg/l IAA + 2.0 mg/l NAA + 0.4 mg/l KN + 2% sucrose
E. 2.0 mg/l IAA + 2.0 mg/l NAA + 2.0 mg/l KN + 2% sucrose

Medium	Fresh weight (mg/culture)	Dry weight (mg/culture)	Phenolics (mg%)	Phenolics (mg/culture)
A	3950.9 (±114.2)	216.32 (±10.7)	1.12	2.61
B	10903.38 (±366.0)	336.2 (±22.4)	1.84	6.18
C	4154.7 (±198.7)	254.62 (±20.0)	2.16	5.49
D	16472.7 (±435.1)	368.9 (±35.5)	1.22	4.50
E	13637.2 (±496.8)	654.4 (±38.3)	1.87	12.23

Data represents an average of 5 replicates.

Figures in parenthesis are standard error.

Fig. 19. Histogram depecting growth (fresh and dry wt.) and polyphenol accumulation in 30 day old cultures of diploid tobacco callus cultured on :

A : MS + IAA (2.0 mg/l) + KN (0.4 mg/l) + Sucrose (1%)

B : " + " + " + Sucrose (2%)

C : " + " + " + Sucrose (4%)

D : " + IAA (2.0 mg/l) + NAA (2.0 mg/l) + KN (0.4 mg/l) + Sucrose (2%)

E : " + " + " + KN (2.0 mg/l) + "

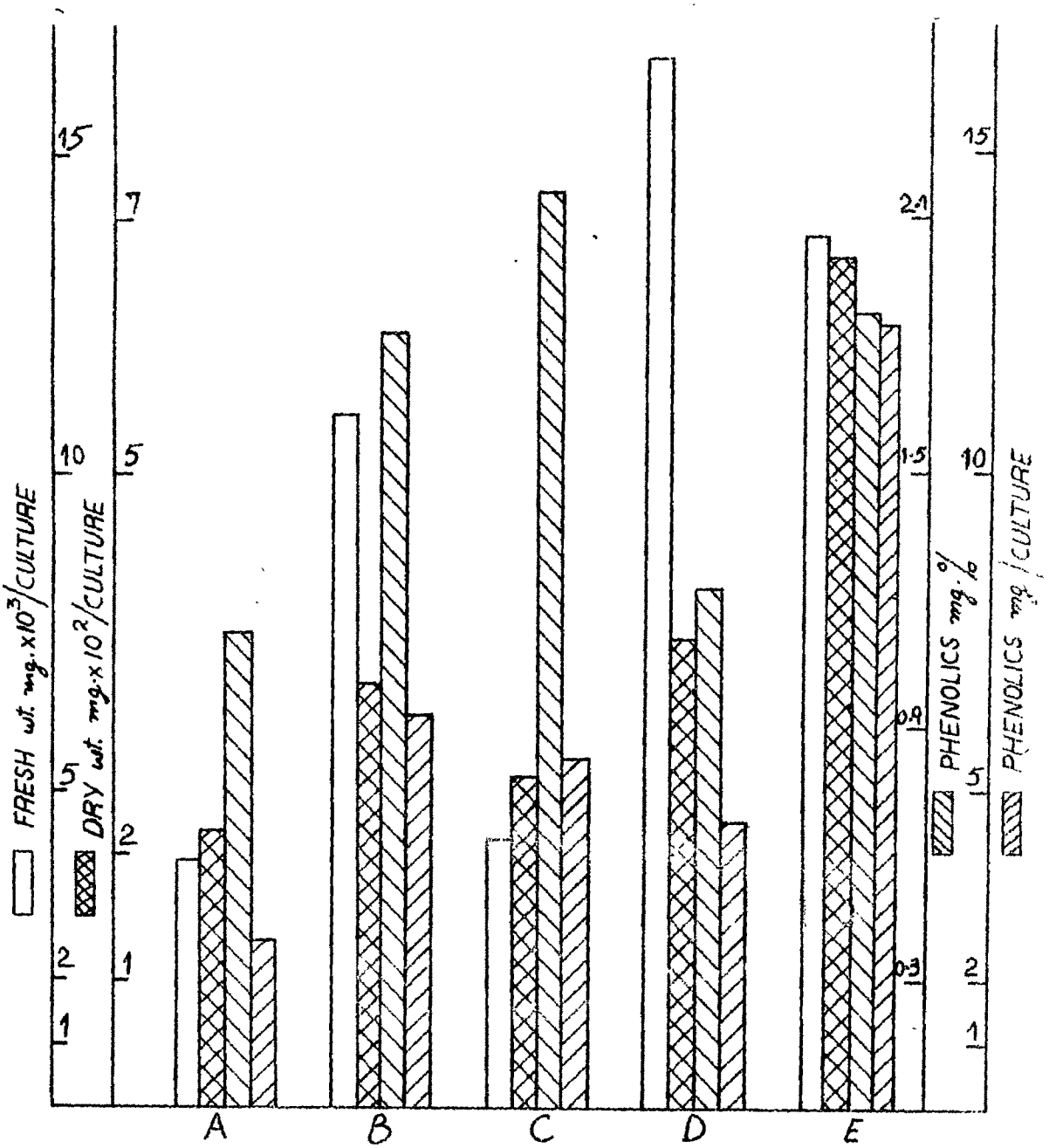

FRESH wt. mg.x10³/CULTURE
DRY wt. mg.x10²/CULTURE
PHENOLICS mg.%
PHENOLICS mg./CULTURE
A
B
C
D
E

FIG. 19

the MS basal medium with 2% sucrose the fresh weight increased 45.45 fold and dry weight by 54.51 fold after 30 days of culture. The accumulation of phenolics was to the tune of 1.87 mg% and 12.23 mg/culture on the second medium, whereas on the first medium the phenolic accumulation was 1.22 mg% and 4.5 mg/culture (Fig. 20, Table 7).

Section B-II. Growth and Accumulation of Phenolic Compounds in Haploid Callus Cultures of *Nicotiana tabacum* L. var. Anand-2.

B - II. Growth and accumulation of phenolic compounds in haploid callus cultures

To study the hormonal influence on growth and the accumulation of phenolic compounds, callus masses weighing 300±30 mg by fresh weight (12±3 mg dry weight) were incubated on 40 ml agar medium in continuous light at 26±2°. The callus tissues were incubated on MS basal medium for 1 week before hormonal supplements were made to minimize any carry over effects. The tissues were harvested on the completion of 30 days in culture and analysed for fresh and dry weight increases, and also for phenolic accumulation. The results are presented in Tables 8 - 10 and Figures 21 - 29.

Expt. 8. Influence of Auxins

Auxins — 2,4-D, IAA, NAA and IBA — were incorporated into the MS basal medium containing 2 per cent sucrose. Each auxin was used at the concentrations of 0.5, 2.0 and 4.0 mg/l in the MS medium to study their effect on growth and phenolic accumulation after 30 days in culture. Callus tissues cultured on MS basal medium acted as the control.

On MS basal medium supplemented with 2 per cent sucrose only, the callus tissues registered 1.52 and 2.85 fold increas in fresh and dry weights respectively. The phenolic content was 1.35 mg% and 0.46 mg/culture (Fig. 21, Table 8).

Fig. 20. Histogram depicting growth (fresh and dry wt.) and polyphenol accumulation in 30 day old cultures of haploid tobacco callus cultured on :

MS basal + 2% sucrose

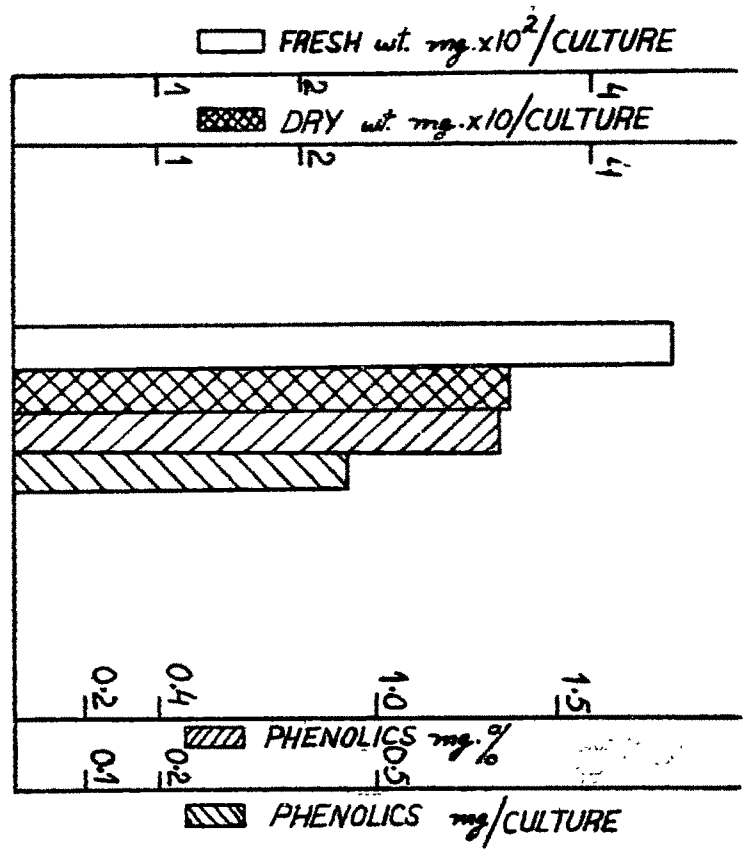

FRESH wt. mg.x10²/CULTURE
1
1.2
1.4
DRY wt. mg.x10/CULTURE
1
1.2
1.4
0.2
0.4
1.0
1.5
PHENOLICS mg.%
0.1
0.2
0.5
PHENOLICS mg/CULTURE

Fig. 20

Fig. 21. Histogram depicting growth (fresh and dry wt.) and polyphenol accumulation in 30 day old cultures of haploid tobacco callus cultured on :

A : MS basal + 2% sucrose

B : MS + 0.5 mg/l 2,4-D + 2% sucrose

C : MS + 2.0 mg/l 2,4-D + 2% sucrose

D : MS + 4.0 mg/l 2,4-D + 2% sucrose

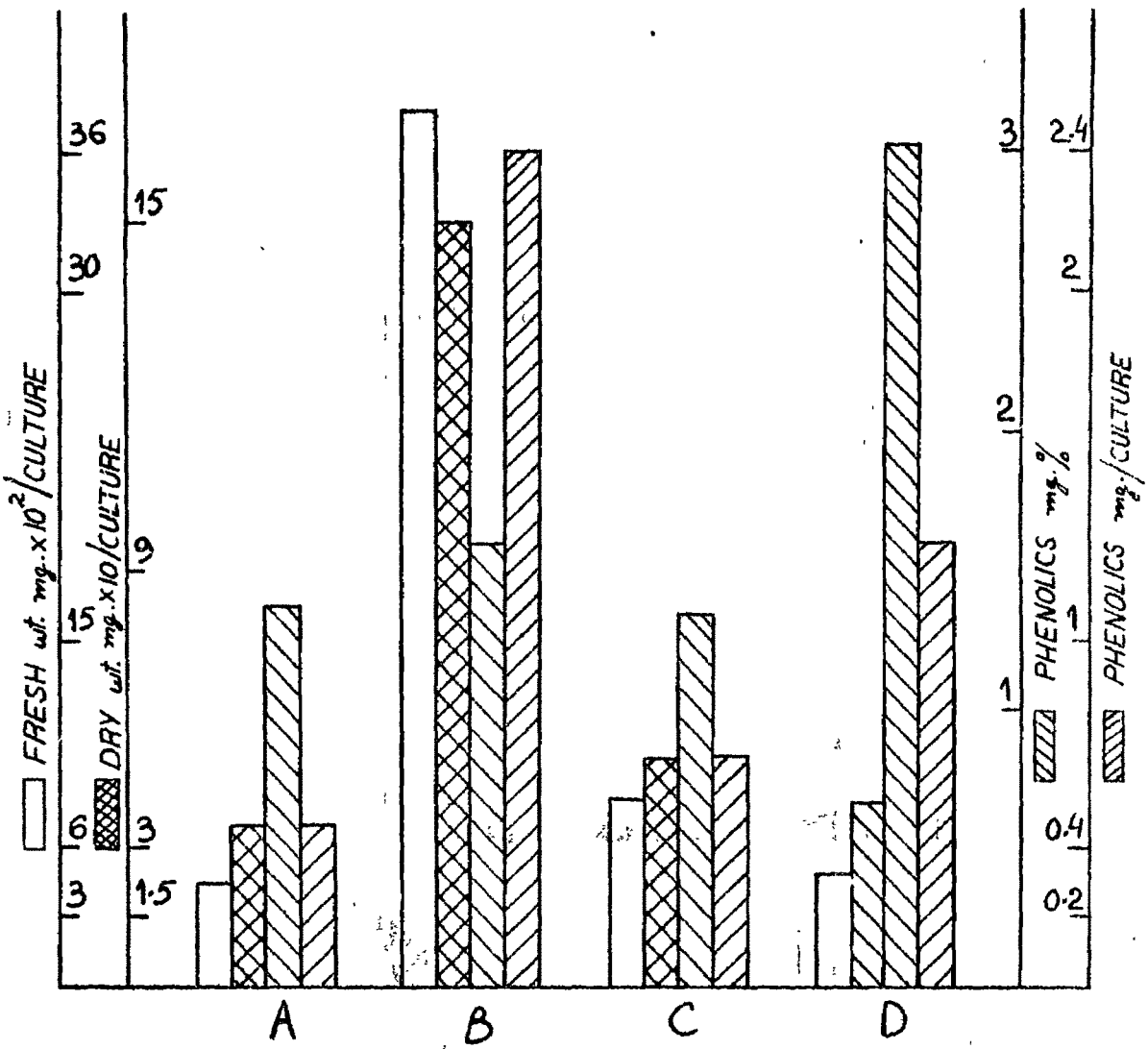

FRESH wt. mg. x 10²/CULTURE
DRY wt. mg. x 10/CULTURE
PHENOLICS mg. %
PHENOLICS mg./CULTURE
36
30
15
6
3
1·5
15
9
3
2·4
2
2
1
1
0·4
0·2
A
B
C
D

FIG. 21

Incorporation of 2,4-D (0.5 mg/l) resulted in 12.6 and 12.54 fold increase in fresh and dry weights respectively. With the increase of 2,4-D concentration to 2.0 and 4.0 mg/l the fresh weight increases were less significant, being 2.7 and 1.53 fold respectively. The dry weight of the callus tissue on these two media registered a 4.09 and 3.47 fold increase. On percentage basis, 4.0 mg/l 2,4-D facilitated maximum phenolic accumulation being 3.05 mg%. However, it was 0.5 mg/l 2,4-D which gave maximum yield of phenolics on per culture basis, being 2.408 mg/culture (Fig. 22, Table 8). The callus tissues on the 2,4-D containing media were hard, compact and turned brown.

Of the three concentrations of IAA incorporated into MS basal medium, maximum growth was supported by 2.0 mg/l, followed closely by 4.0 and 0.5 mg/l IAA. The fold-wise increase in fresh weights was 2.31, 1.97 and 1.83 respectively. On dry weight basis, it was 4.0 mg/l IAA followed by 2.0 and 0.5 mg/l. The fold-wise increases were 4.21, 4.13 and 3.68 respectively. The phenolic accumulation on mg percentage and mg/culture basis was maximum on 4.0 mg/l IAA containing medium, the respective values being 2.30 mg% and 1.16 mg/culture. 0.5 and 2.0 mg/l IAA each supported 1.8 mg% phenolic accumulation and the mg/culture accumulation was 0.79 and 0.89 mg respectively (Fig. 23, Table 8). The callus tissues

Table : 8. Auxin effect on growth and accumulation of phenolic compounds in haploid callus of Nicotiana tabacum L.

Inoculum : 300±30 mg tissue by fresh weight (12±3 mg dry weight) on 40 ml of MS medium supplemented with 2% sucrose and different auxins.

Incubation : 30 days in light at 25±2°

Influence of auxins

Medium	Auxin (mg/l)	Fresh weight (mgm/culture)	Dry weight (mg/culture)	Phenolics (mg%)	Phenolics (mg/cult.)
MS (Basal) + 2% sucrose	-	457.6 (±42)	34.3 (±8)	1.35	0.463
"	2,4-D (0.5 mg/l)	3782.8 (±43.1)	150.5 (±15)	1.60	2.408
"	2,4-D (2.0 mg/l)	811.0 (±10.7)	49.13 (±7)	1.35	0.663
"	2,4-D (4.0 mg/l)	461.2 (±43)	41.66 (±9)	3.05	1.270
"	IAA (0.5 mg/l)	550.2 (±59)	44.26 (±7.9)	1.80	0.796
"	IAA (2.0 mg/l)	694.46 (±25.9)	49.64 (±5.7)	1.80	0.893
"	IAA (4.0 mg/l)	591.78 (±13.0)	50.6 (±3.2)	2.30	1.163
"	NAA (0.5 mg/l)	1103.58 (±230)	94.08 (±18.5)	2.05	1.928
"	NAA (2.0 mg/l)	4209.46 (±130.3)	181.63 (±31.6)	2.05	3.723
"	NAA (4.0 mg/l)	809.8 (±24.5)	60.51 (±7.2)	1.60	0.968
"	IBA (0.5 mg/l)	677.8 (±14.2)	43.46 (±8.2)	1.10	0.478
"	IBA (2.0 mg/l)	1240.0 (±107.3)	48.31 (±7.7)	0.9	0.434
"	IBA (4.0 mg/l)	764.06 (±81.8)	57.98 (±7.5)	2.05	1.188

Data represents an average of 5 replicates.

Figures in parenthesis represent standard error.

Fig. 22. Histogram depicting growth (fresh and dry wt.) and polyphenol accumulation in 30 day old cultures of haploid tobacco callus cultured on :

A : MS basal + 2% sucrose

B : MS + 0.5 mg/l IAA + 2% sucrose

C : MS + 2.0 mg/l IAA + 2% sucrose

D : MS + 4.0 mg/l IAA + 2% sucrose

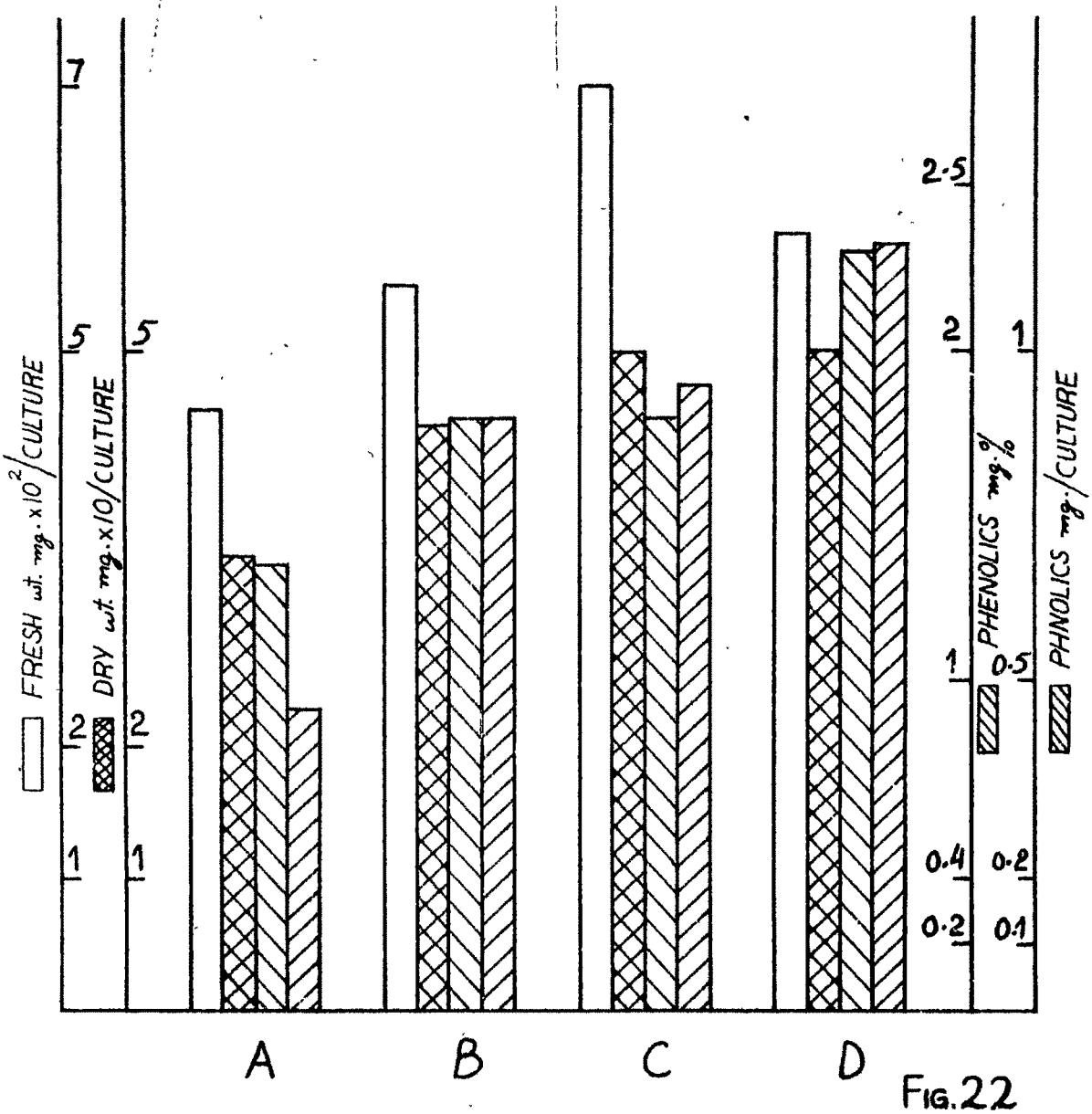

FRESH wt. mg. x 10²/CULTURE
DRY wt. mg. x 10/CULTURE
PHENOLICS mg.%
PHNOLICS mg./CULTURE
7
5
2
1
5
2
1
2·5
2
1
1
0·5
0·4
0·2
0·2
0·1
A
B
C
D

FIG. 22

Fig. 23. Histogram depicting growth (fresh and dry wt.) and polyphenol accumulation in 30 day old cultures of haploid tobacco callus cultured on :

A : MS basal + 2% sucrose

B : MS + 0.5 mg/l NAA + 2% sucrose

C : MS + 2.0 mg/l NAA + 2% sucrose

D : MS + 4.0 mg/l NAA + 2% sucrose

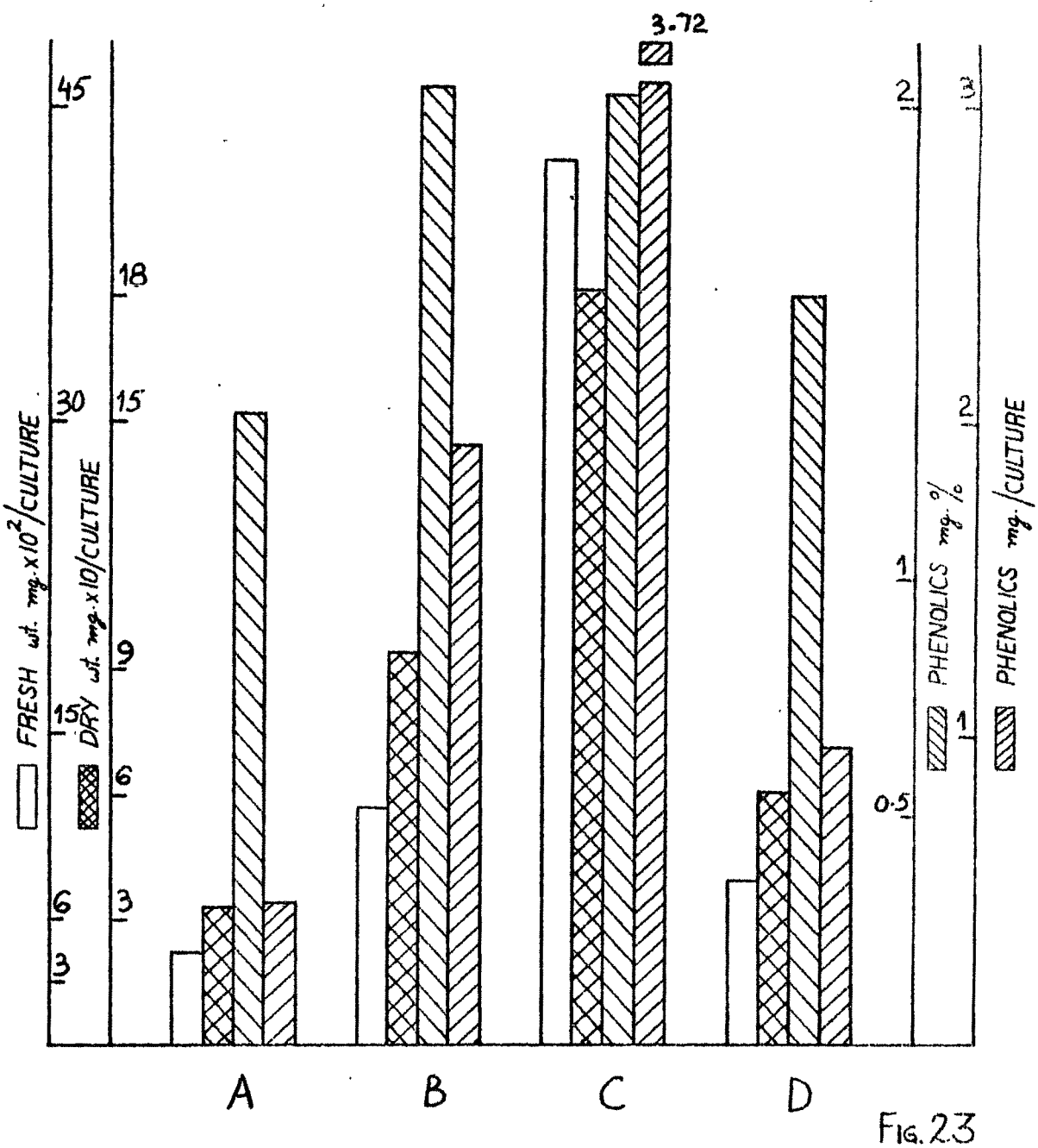

3·72
45
30
15
6
3
18
15
9
6
3
2
1
1
0·5
FRESH wt. mg.×10²/CULTURE
DRY wt. mg.×10/CULTURE
PHENOLICS mg.%
PHENOLICS mg./CULTURE
A
B
C
D

FIG. 23

on IAA containing media were green, soft, nodular and highly friable.

Incorporation of NAA (2.0 mg/l) supported maximum growth of the callus tissues on both fresh and dry weight basis. The fresh weight increased 14.03 fold and the dry weight 15.13 fold. 0.5 mg/l NAA was more effective than 4.0 mg/l NAA in supporting growth. The former showing 3.67 and the latter 2.69 fold increase in fresh weight. The corresponding dry weight increases were 7.84 and 5.04 fold respectively. 2.05 mg % phenolics accumulated in callus tissues on 0.5 and 2.0 mg/l NAA and it decreased to 1.6 mg % with the increase of NAA level to 4.0 mg/l. On mg/culture basis maximum accumulation of phenolics was obtained on 2.0 mg/l NAA, the yield being 3.72 mg/culture followed by 0.5 and 4.0 mg/l NAA providing with 1.92 and 0.96 mg/culture of phenolics respectively (Fig. 24, Table 8). The callus tissues grown on NAA containing media were green in colour, highly plastic and watery by constitution.

Among the three concentrations of IBA (0.5, 2.0 and 4.0 mg/l) tested for growth and accumulation of phenolic compounds in callus tissues, it was found that 2.0 mg/l IBA gave maximum increase in fresh weight (4.13 fold) followed by 4.0 mg/l IBA (2.54 fold) and 0.5 mg/l IBA (2.25 fold).

Fig. 24. Histogram depicting growth (fresh and dry wt.) and polyphenol accumulation in 30 day old cultures of haploid tobacco callus cultured on :

A : MS basal + 2% sucrose

B : MS + 0.5 mg/l IBA + 2% sucrose

C : MS + 2.0 mg/l IBA + 2% sucrose

D : MS + 4.0 mg/l IBA + 2% sucrose

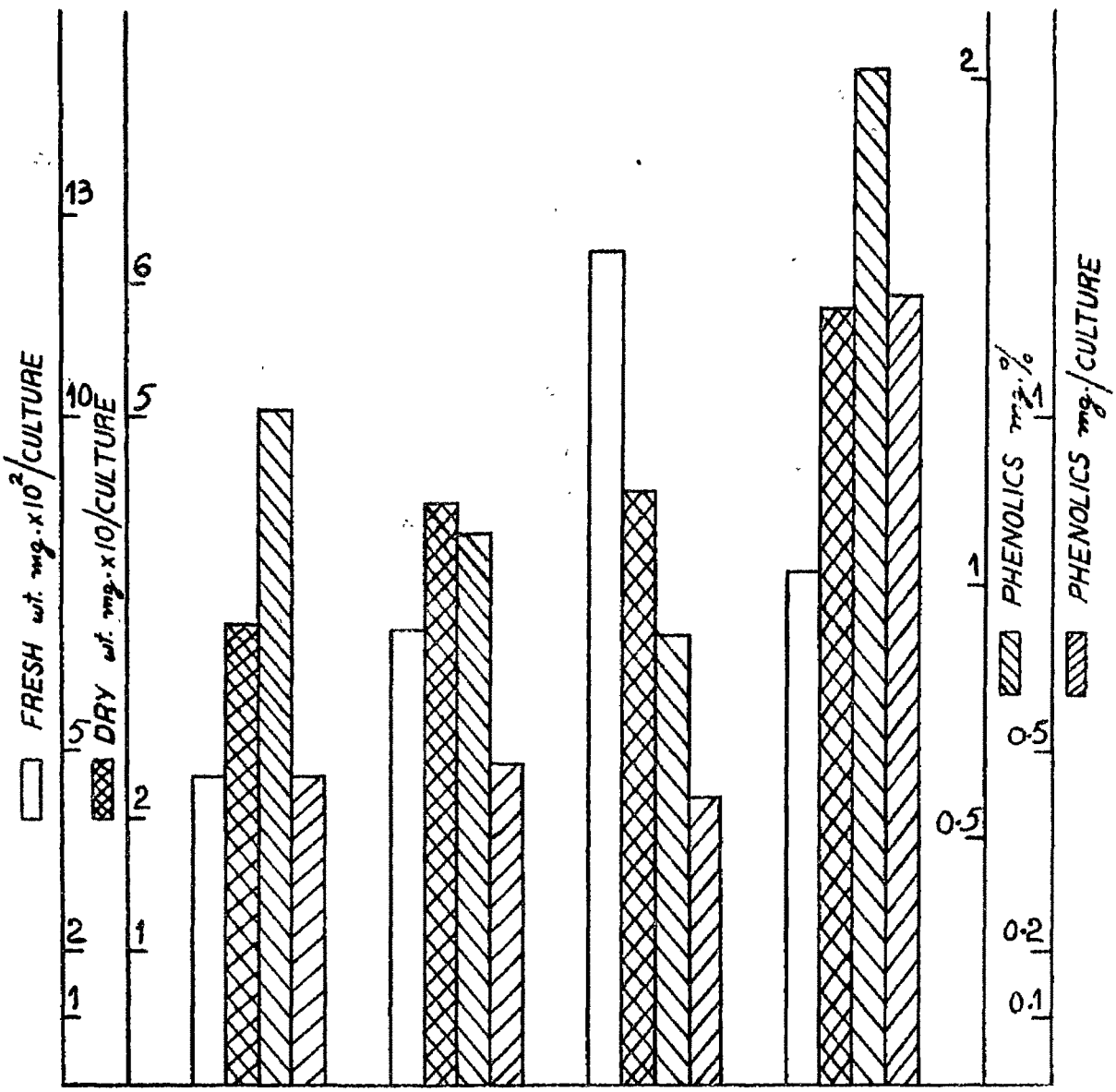

FRESH wt. mg.x10²/CULTURE
DRY wt. mg.x10/CULTURE
PHENOLICS mg.%
PHENOLICS mg./CULTURE
13
10
6
5
2
1
2
1
2
1
0.5
0.5
0.2
0.1
A
B
C
D

FIG. 24

4.0 mg/l IBA gave maximum increase in dry weight (4.83). With the reduction of IBA concentration in the medium the dry weight also dropped. 2.0 mg/l IBA gave an increase of 4.02 fold and 0.5 mg/l gave 3.62 fold increases in dry weights. 4.0 mg/l IBA also proved to be more effective than its other two concentrations in facilitating phenolic accumulation. The corresponding values were 2.05 mg % for 4.0 mg/l IBA, 0.9 mg % for 2.0 mg/l IBA and 1.1 mg % for 0.5 mg/l IBA. Identical trend was followed for the accumulation of phenolic compounds on mg/culture basis. The values being 1.18, 0.43 and 0.47 mg/culture for 4.0, 2.0 and 0.5 mg/l IBA (Fig. 25, Table 8). The callus tissues were green, compact and hard.

Expt. 9. Influence of Cytokinins

Prior to setting-up of the experiment the callus tissues were incubated for one week on MS basal medium free of any auxin or cytokinin to eliminate any carry over effect. Callus masses 300±30 mg by fresh weight (12±3 mg dry weight) were transferred onto 40 ml MS medium supplemented (singly) with 0.1, 0.4 and 1.0 mg/l of KN and 6-dimethylaminopurine. The cultures were incubated for 30 days in continuous light at 25±2°. A fixed number of replicates were harvested at the end of 30 days and analysed for growth measurements and phenolic contents.

Table : 9. Growth and accumulation of phenolic compounds in haploid callus of Nicotiana tabacum L.

Inoculum : 300±30 mg callus tissue by fresh weight (12±3 mg dry weight) on 40 ml of MS basal medium with 2% sucrose, and various levels of cytokinins and GA_3.

Incubation : 30 days in light at 25±2°.

Phytohormone	Concentration (mg/l)	Fresh weight (mg/culture)	Dry weight (mg/culture)	Phenolics (mg%)	Phenolics (mg/culture)
-	-	457.6 (±42)	34.3 (±8)	1.35	0.46
KN	0.1	1339.0 (±34.7)	73.7 (±3.3)	0.90	0.63
"	0.4	1421.85 (±38.2)	91.8 (±4.6)	0.45	0.41
"	1.0	1898.58 (±43.5)	185.8 (±3.7)	0.45	0.83
MAP	0.1	357.83 (±29.8)	28.2 (±4.2)	0.45	0.12
"	0.4	290.9 (±30.1)	14.4 (±3.0)	0.2	0.02
"	1.0	389.8 (±46.8)	31.3 (±4.6)	0.45	0.14
GA_3	10.0	465.8 (±36.6)	26.23 (±2.7)	0.82	0.21
"	50.0	406.73 (±29.8)	24.76 (±4.0)	0.76	0.18
"	100.0	815.85 (±92.1)	50.3 (±9.3)	0.58	0.29

Data represents an average of 5 replicates.
Figures in parenthesis represent standard error.

Fig. 25. Histogram depicting growth (fresh and dry wt.) and polyphenol accumulation in 30 day old cultures of haploid tobacco callus cultured on :

A : MS basal + 2% sucrose

B : MS + 0.1 mg/l KN + 2% sucrose

C : MS + 0.4 mg/l KN + 2% sucrose

D : MS + 1.0 mg/l KN + 2% sucrose

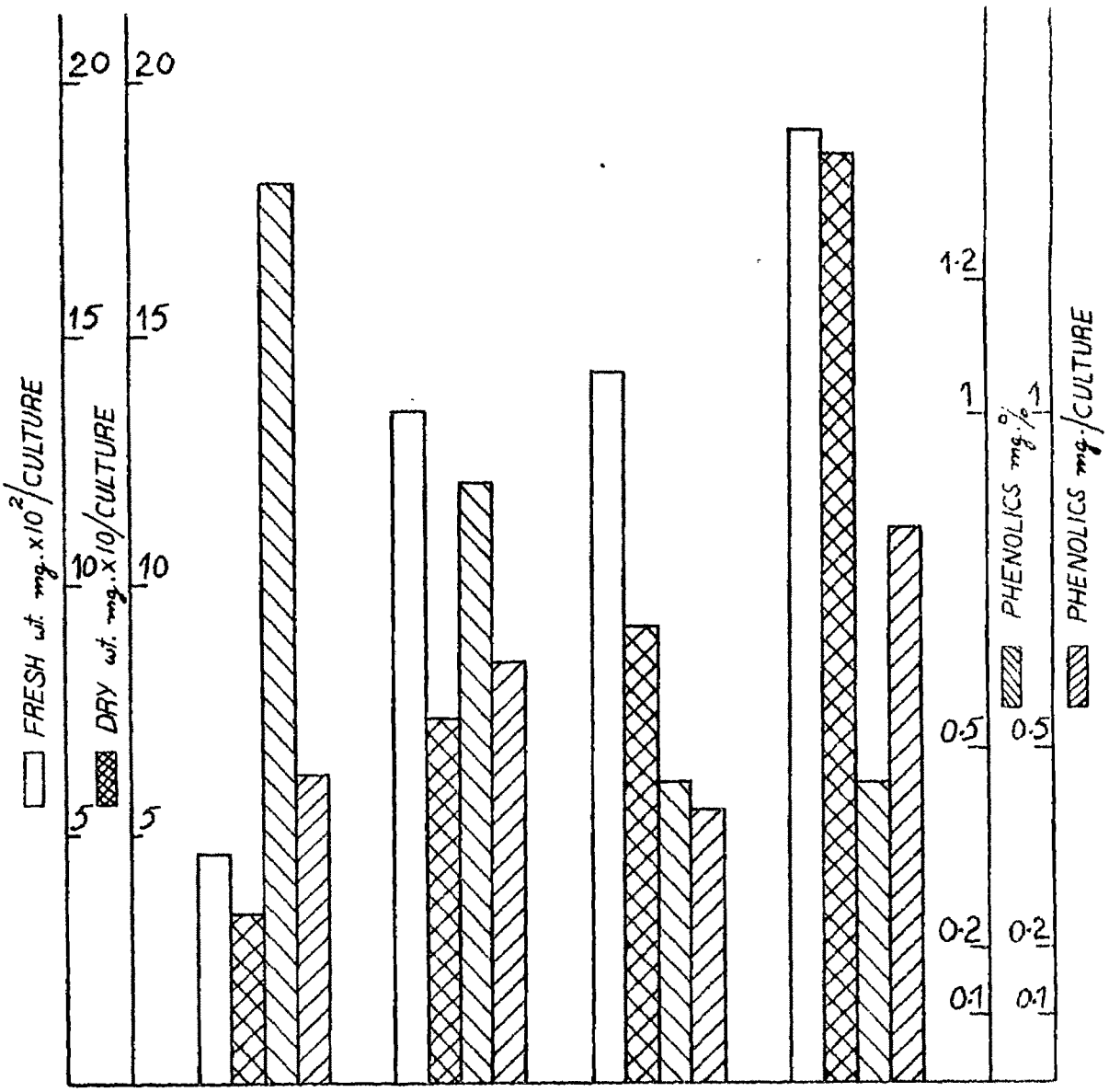

FRESH wt. mg. x10²/CULTURE
DRY wt. mg. x10/CULTURE
PHENOLICS mg.%
PHENOLICS mg./CULTURE
20
15
10
5
1·2
1
0·5
0·2
0·1
A
B
C
D

FIG. 25

Kinetin at all the three concentrations tested (0.1, 0.4 and 1.0 mg/l) enhanced the growth of haploid callus. The respective fold-wise increase in fresh weights were 4.46, 4.73 and 6.32. The dry weight on the two lower concentrations did not increase as effeciently as at the higher level. The actual fold-wise values being 6.14 and 7.65. On 1.0 mg/l KN the dry weight increased by 15.48 fold. At 0.4 and 1.0 mg/l KN only 0.45 mg% each, of phenolics accumulated. The mg per culture yield of phenolics was 0.41 and 0.83 mg/culture respectively. Maximum phenolic accumulation of 0.90 mg% was supported by 0.1 mg/l KN. The total phenolic accumulation on culture basis was 0.46 mg/culture (Fig. 26, Table 9).

The callus tissues grown on 0.1 and 1.0 mg/l KN was highly nodular, compact and turned brown during the culture period. The tissue was, however, green and friable when grown on 0.4 mg/l KN.

The other cytokinin tested, 6-dimethylaminopurine (MAP), unlike KN did not show any pronounced effect on growth of the tissues. The fresh weights increased 1.19 and 1.29 fold with the callus tissues cultured on 0.1 and 1.0 mg/l MAP respectively. No increase was noticed in fresh weight of the callus tissues on 0.4 mg/l MAP. 0.1 and 1.0 mg/l MAP

Fig. 26. Histogram depicting growth (fresh and dry wt.) and polyphenol accumulation in 30 day old cultures of haploid tobacco callus cultures on :

A : MS basal +2% sucrose

B : MS + 0.1 mg/l MAP + 2% sucrose

C : MS + 0.4 mg/l MAP + 2% sucrose

D : MS + 1.0 mg/l MAP + 2% sucrose.

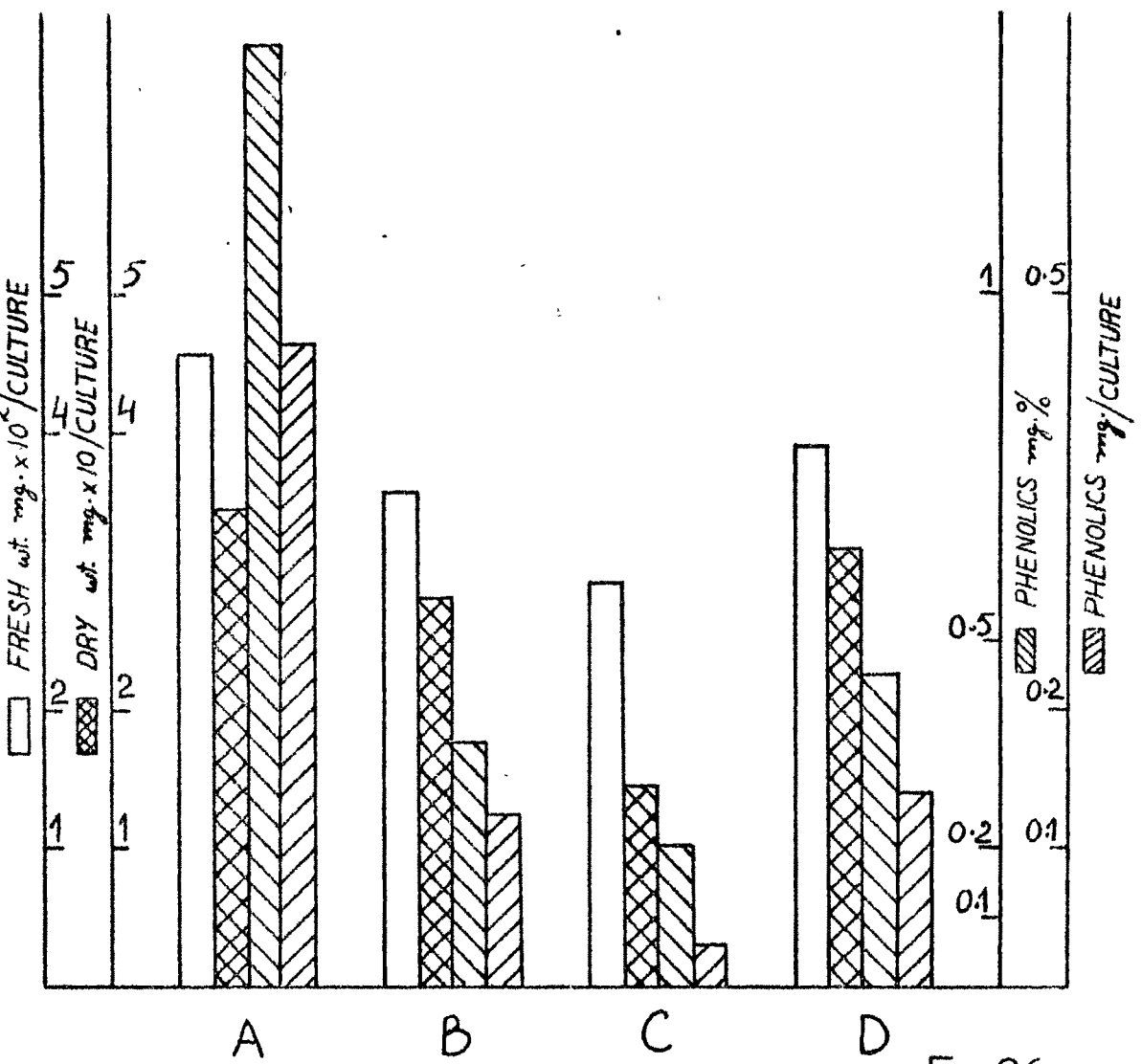

FRESH wt. mg. x 10²/CULTURE
DRY wt. mg. x 10/CULTURE
PHENOLICS mg.%
PHENOLICS mg./CULTURE
5
4
2
1
0·5
0·2
0·1
A
B
C
D

FIG. 26

gave 2.35 and 2.6 fold increase in dry weights respectively. 0.4 mg/l MAP did not bring about any increase in the dry weight. The phenolic accumulation in callus tissues was 0.45 mg % and 0.126 mg/culture on 0.1 mg/l MAP; 0.2 mg % and 0.028 mg/culture on 0.4 mg/l MAP, and 0.45 mg % and 0.14 mg/culture on 1.0 mg/l MAP (Fig. 27, Table 9).

The callus tissues were dark brown and compact when grown on the above concentrations of MAP.

Expt. 10. Influence of GA_3

To study the influence of Gibberellic Acid (GA_3) on growth and accumulation of phenolic compounds, it was incorporated into the MS basal medium in three concentrations of 10, 50 and 100 mg/l.

Of the three GA_3 concentrations the lower two (10 and 50 mg/l) supported only marginal increases in the fresh weight, the fold-wise increases being 1.55 and 1.35 respectively. GA_3 at 100 mg/l brought about an increase of 2.71 fold in fresh weight. Though the fresh weight varied over a wide range of 1.35 - 2.71 fold increases, the dry weights at all the three concentrations remained more or less alike, the fold-wise increases being 2.18, 2.06 and 4.19 at 10, 50 and 100 mg/l GA_3 respectively. The phenolic accumulation in

Fig. 27. Histogram depicting growth (fresh and dry wt.) and polyphenol accumulation in 30 day old cultures of haploid tobacco callus cultured on :

A : MS basal + 2% sucrose

B : MS + 10 mg/l GA_3 + 2% sucrose

C : MS + 50 mg/l GA_3 + 2% sucrose

D : MS +100 mg/l GA_3 + 2% sucrose

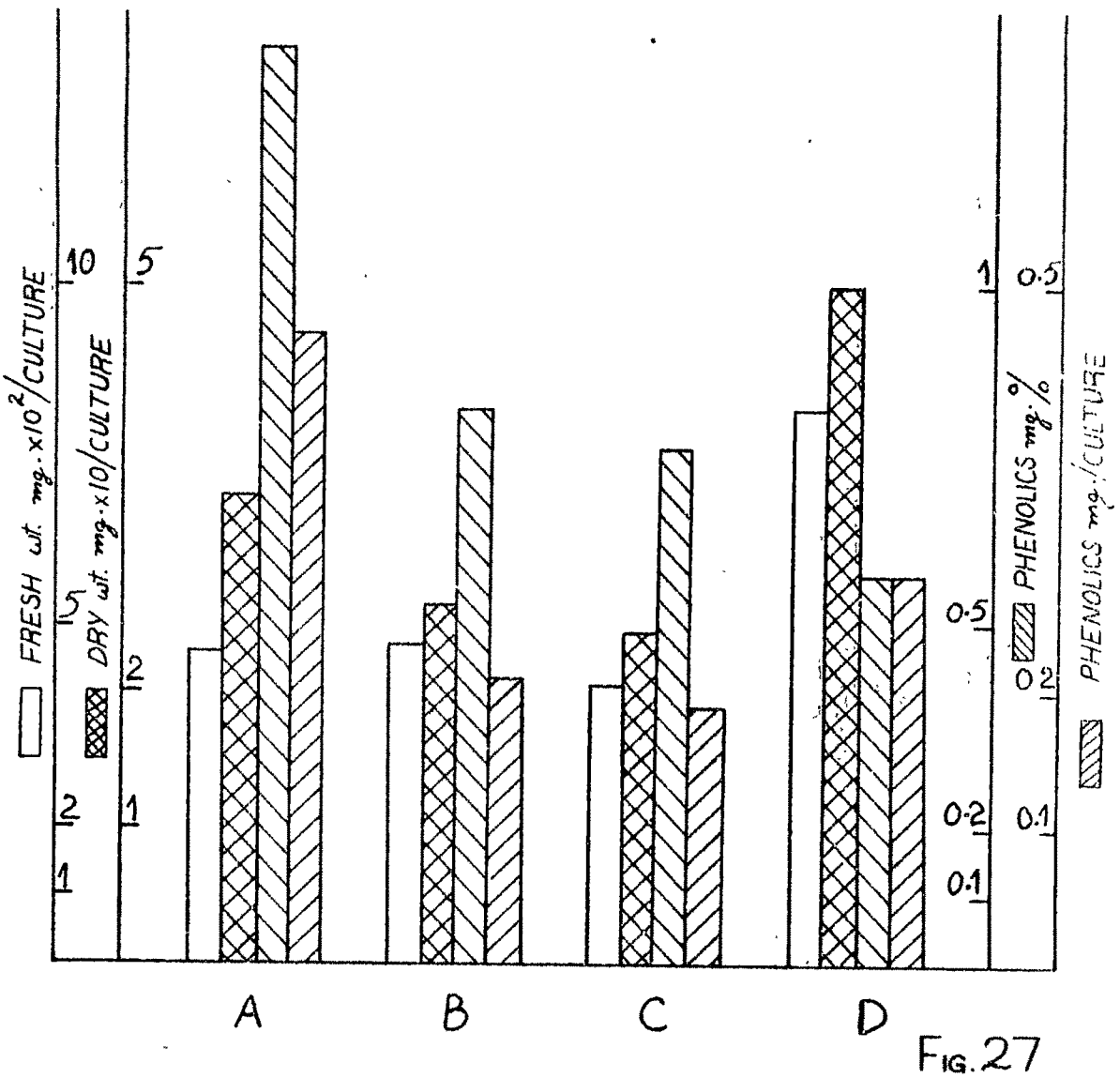

FRESH wt. mg. x10²/CULTURE
DRY wt. mg. x10/CULTURE
PHENOLICS mg.%
PHENOLICS mg./CULTURE
10
5
2
1
1
0.5
0.2
0.1
A
B
C
D

FIG. 27

Table : 10. Effect of sucrose level in presence of auxin and kinetin on growth and accumulation of phenolic compounds in haploid callus of Nicotiana tabacum.

Inoculum : 300±30 mg tissue by fresh weight (12±3 mg dry weight) on 40 ml MS medium supplemented with :

A. 2.0 mg/l IAA + 0.4 mg/l KN + 1% sucrose
B. 2.0 mg/l IAA + 0.4 mg/l KN + 2% sucrose
C. 2.0 mg/l IAA + 0.4 mg/l KN + 4% sucrose
D. 2.0 mg/l IAA + 2.0 mg/l NAA + 0.4 mg/l KN + 2% sucrose
E. 2.0 mg/l IAA + 2.0 mg/l NAA + 2.0 mg/l KN + 2% sucrose

Medium	Fresh weight (mg/culture)	Dry weight (mg/culture)	Phenolics (mg%)	Phenolics (mg/culture)
A	1471.26 (±43.2)	93.6 (±6.6)	0.45	0.42
B	1199.0 (±57.8)	99.2 (±9.2)	0.65	0.64
C	1047.6 (±44.1)	96.8 (±4.1)	1.10	1.06
D	17439.43 (±261.3)	469.9 (±18.1)	0.66	3.10
E	15600.32 (±344.9)	587.4 (±22.8)	1.12	6.57

Data represents an average of 5 replicates.

Figures in parenthesis are standard error.

callus tissues was 0.21 mg/culture and 0.82 mg% on 10.0 mg/l GA_3; 0.18 mg/culture and 0.76 mg% on 50.0 mg/l GA_3 and 0.29 mg/culture and 0.58 mg% on 100.0 mg/l GA_3 (Fig. 28, Table 9).

Expt. 11. Influence of various sucrose levels in combination with auxins and kinetin.

Auxins and kinetin were taken in combination at different levels of sucrose to study their influence on the growth and phenolic accumulation in haploid callus tissues of N. tabacum. The callus tissues were kept for one week on MS basal medium prior to its inoculation on media containing phyto-hormones to minimize any carry over effect. The callus tissues were incubated over a period of 30 days before analysis for growth and phenolics.

IAA (2.0 mg/l) and KN (0.4 mg/l) were taken in combination with sucrose added in concentration range of 1 - 4%. Increasing sucrose concentrations resulted in decreasing growth values on fresh weight basis. The fold-wise fresh weight increases on 1, 2 and 4% sucrose containing media were 4.9, 3.96 and 3.49 respectively. On dry weight basis the fold-wise increases were 7.8, 8.26 and 8.06 respectively. Increasing sucrose levels led to increasing accumulation of phenolics. The values of phenolic accumulation on 1, 2 and 4% sucrose containing medium were 0.45, 0.65 and 1.10 mg% respectively. On culture basis the

Fig. 28. Histogram depicting growth (fresh and dry wt.) and polyphenol accumulation in 30 day old cultures of haploid tobacco callus cultured on :

A : MS basal + 2% sucrose

B : MS + IAA (2.0 mg/l) + KN (0.4 mg/l) + sucrose (1%)

C : " + " + " + sucrose (2%)

D : " + " + " + sucrose (4%)

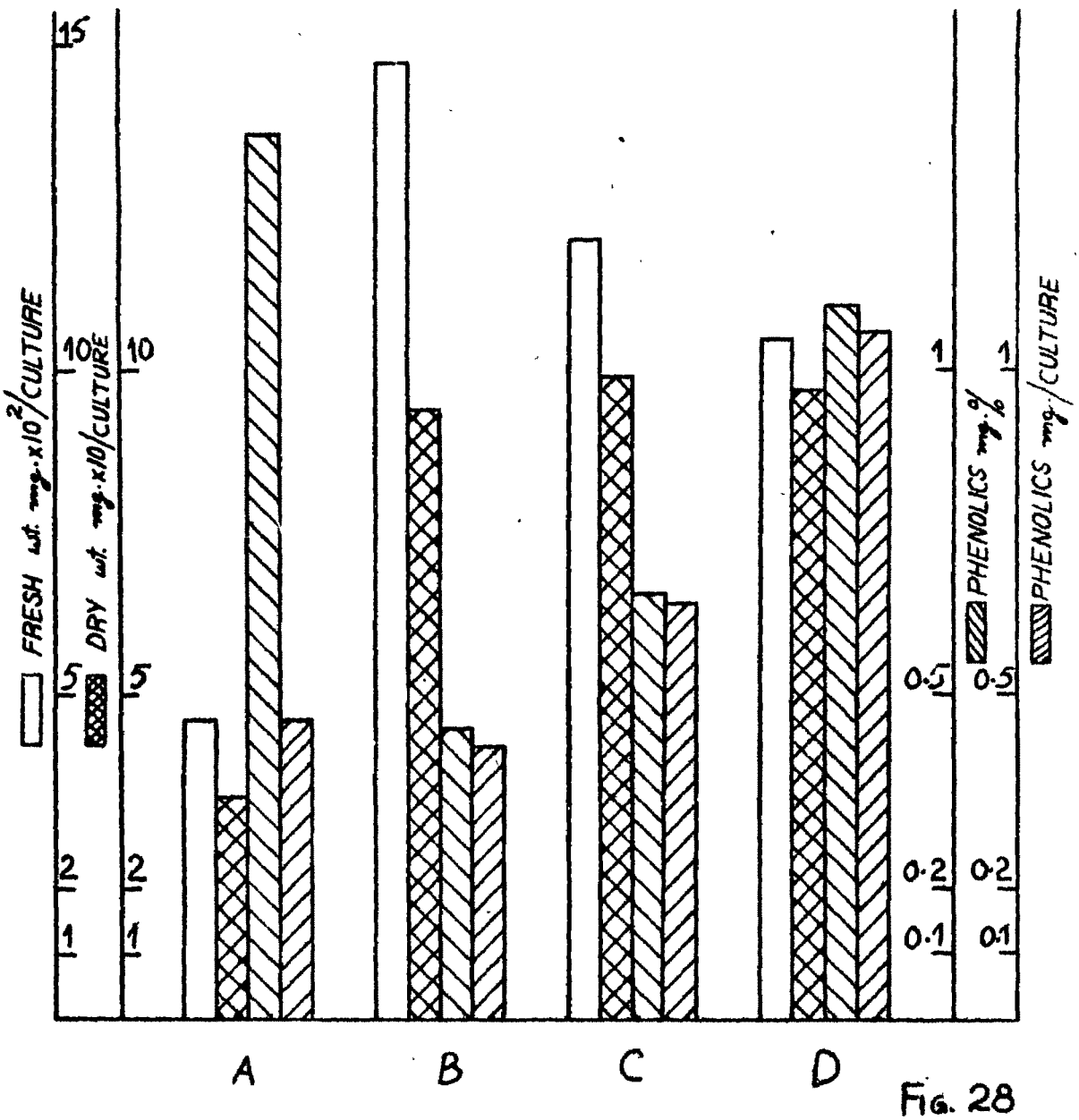

FRESH wt. mg.x10²/CULTURE
DRY wt. mg.x10/CULTURE
PHENOLICS %
PHENOLICS mg./CULTURE
15
10
5
2
1
0.5
0.2
0.1
A
B
C
D

Fig. 28

Fig. 29. Histogram depicting growth (fresh and dry wt.) and polyphenol accumulation in 30 day old cultures of haploid tobacco callus cultured on :

A : MS basal + 2% sucrose

B : MS + IAA (2.0 mg/l) + NAA (2.0 mg/l) + KN (0.4 mg/l) + sucrose (2%)

C : " + " + " + KN (2.0 mg/l) + "

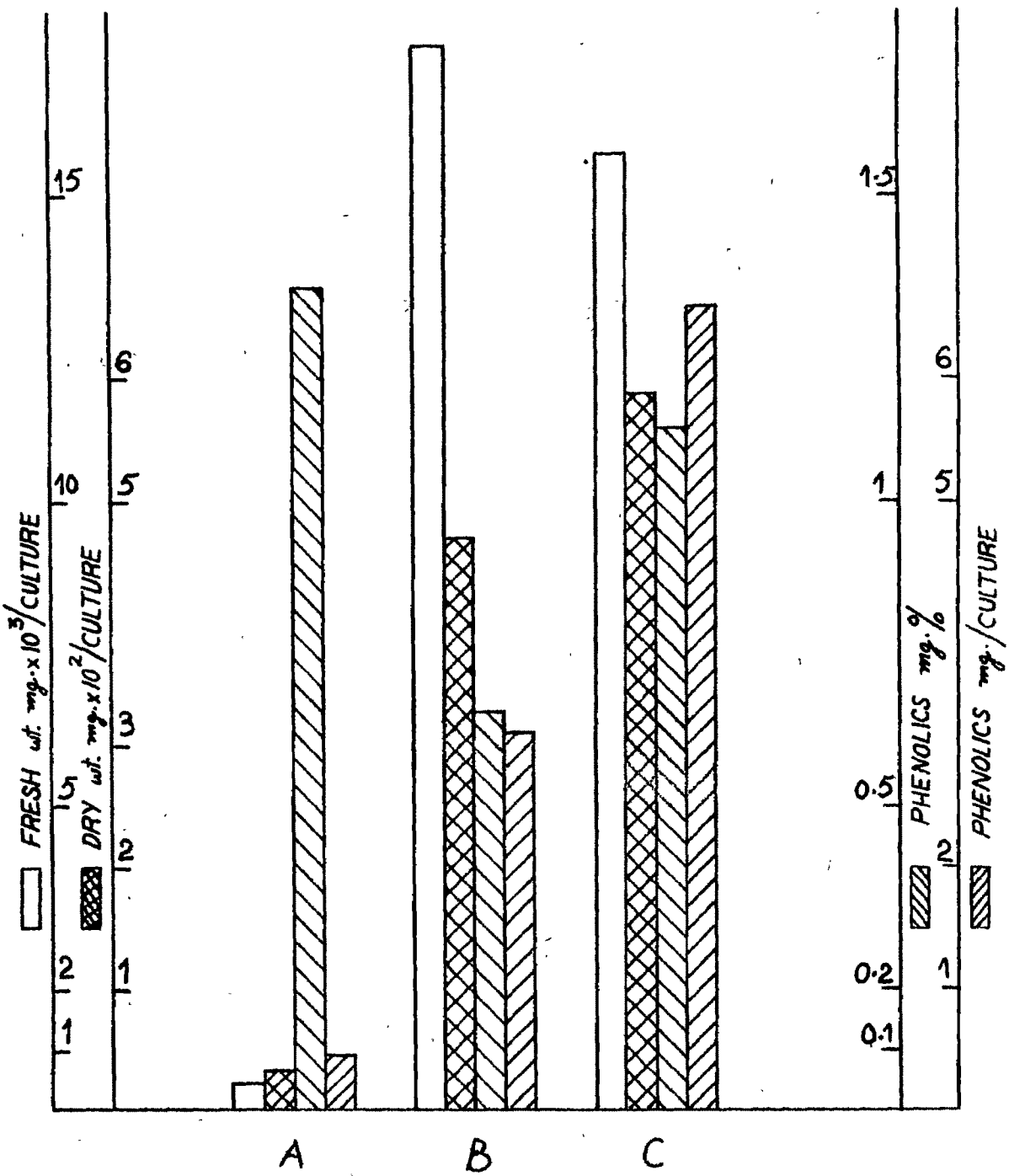

FRESH wt. mg.×10³/CULTURE
DRY wt. mg.×10²/CULTURE
PHENOLICS mg.%
PHENOLICS mg./CULTURE
15
10
5
1
6
3
2
1
1.5
1
0.5
0.2
0.1
6
5
2
1
A
B
C

FIG. 29

corresponding values were 0.42, 0.64 and 1.06 mg/culture (Fig. 29, Table 10).

In another experiment auxins, IAA and NAA were taken in combination with KN. Two combinations tried were 2.0 mg/l IAA + 2.0 mg/l NAA + 0.4 mg/l KN and 2.0 mg/l each of IAA, NAA and KN. The hormones were supplemented into MS basal medium containing 2% sucrose. On the first medium the fresh weight increased 58.13 fold and 52.0 fold on the second. Corresponding dry weight increases were 39.15 and 48.95 fold respectively. Maximum phenolic accumulation was supported by the second medium, the values being 1.12 mg% and 6.57 mg/culture. The phenolic content on the first medium was 0.66 mg% and 3.10 mg/culture (Fig. 29, Table 10).

Section C-I : Organogenesis in Diploid Callus Cultures of *Nicotiana tabacum* L.

C - I. Organogenesis in diploid callus cultures of Nicotiana tabacum L.

Callus cultures derived from the floral buds of N. tabacum and maintained on MS medium supplemented with 2.0 mg/l each of IAA, NAA and KN, were later transferred to MS medium containing varying levels of phytohormones, singly and in combinations, and sucrose to evoke morphogenetic responses.

Expt. 12. Influence of IAA on organogenesis

Green, healthy looking callus masses were cultured on MS medium supplemented with a wide range of IAA (0.175 - 4.0 mg/l) and sucrose (1-6%) as shown below :

i) MS + 2% sucrose
ii) MS + 0.175 mg/l IAA + 2% sucrose
iii) " + " + 3% sucrose
iv) " + 0.3 mg/l IAA + 1% sucrose
v) " + " + 2% sucrose
vi) " + " + 3% sucrose
vii) " + " + 6% sucrose
viii) " + 0.5 mg/l IAA + 2% sucrose
ix) " + 2.0 mg/l IAA + 1% sucrose
x) " + " + 2% sucrose

xi) MS + 2.0 mg/l IAA + 3% sucrose

xii) " + " + 6% sucrose

xiii) " + 3.0 mg/l IAA + 1% sucrose

xiv) " + " + 2% sucrose

xv) " + " + 3% sucrose

xvi) " + " + 6% sucrose

xvii) " + 4.0 mg/l IAA + 1% sucrose

xviii) " + " + 2% sucrose

xix) " + " + 3% sucrose

xx) " + " + 6% sucrose

The cultures were incubated at 26±2° in continuous light and periodic observations made. The results are presented in Table 11 and Figures 30 - 38).

Callus tissues cultured on MS basal medium containing 2% sucrose alone served as the control. On this medium shoots were differentiated during the fourth week of culture with 40-50% frequency. Per callus mass 2-10 shoots were differentiated (Fig. 30, Table 11).

On MS medium containing 0.175 mg/l IAA and 2% sucrose, no organogenetic response was obtained even after four weeks of culture. In the same medium the increase of sucrose level to 3%, however, resulted in the differentiation of flower bud like structures (Fig. 31, Table 11).

On the other hand, 0.25 mg/l IAA in combination with 2 or 3% sucrose failed to invoke either shoot or root differentiation.

On MS medium containing 0.3 mg/l IAA and 1% sucrose similarly, no organogenic response was observed. However, with the increase of sucrose level to 2% at the same auxin concentration, solitary shoots were differentiated during the fourth week of culture with about 50% frequency (Fig. 32, Table 11). Further increase of sucrose level to 3% in the same medium, also as in the previous instance, resulted in the differentiation of solitary shoots during the second week of culture with 50% frequency (Fig. 33, Table 11). When 6% sucrose was used in the same IAA (0.3 mg/l) containing medium, there was shift in organogenic response from shoot to root formation. Roots were differentiated during the second week of culture with about 25% frequency (Fig. 34, Table 11).

On MS medium containing 0.5 mg/l IAA and 2% sucrose, one or two roots were differentiated from diploid tobacco callus culture. Roots usually appeared during the fourth week of culture with 25% frequency (Fig. 35, Table 11).

No organogenetic response was observed in diploid tobacco callus tissues cultured on MS medium supplemented with 2.0 mg/l

Fig. 30. Shoot differentiation from diploid tobacco callus on MS medium + 2% sucrose (6 weeks).

Fig. 31. Flower bud like structures differentiate from diploid callus on MS + 0.175 mg/l IAA + 3% sucrose (4 weeks).

Fig. 32. Solitary underdeveloped shoot from diploid callus on MS + 0.3 mg/l IAA + 2% sucrose (4 weeks).

Fig. 33. Solitary well developed shoot from diploid callus on MS + 0.3 mg/l IAA + 3% sucrose (12-15 days).

Fig. 34. Root differentiation from diploid callus on MS + 0.3 mg/l IAA + 6% sucrose (12 days).

Fig. 35. Root differentiation from diploid callus on MS + 0.5 mg/l IAA + 2% sucrose (4 weeks).

FIG. 30.

FIG. 31.

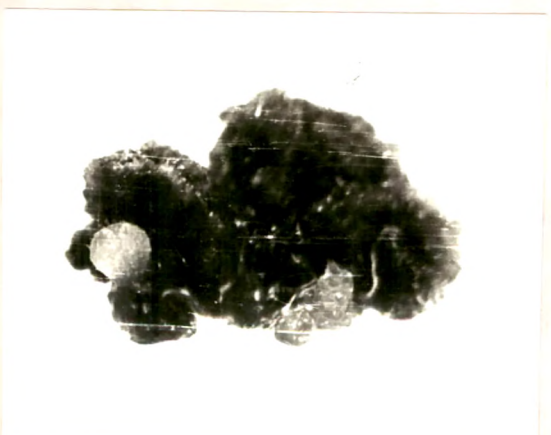


FIG. 32.

FIG. 33.

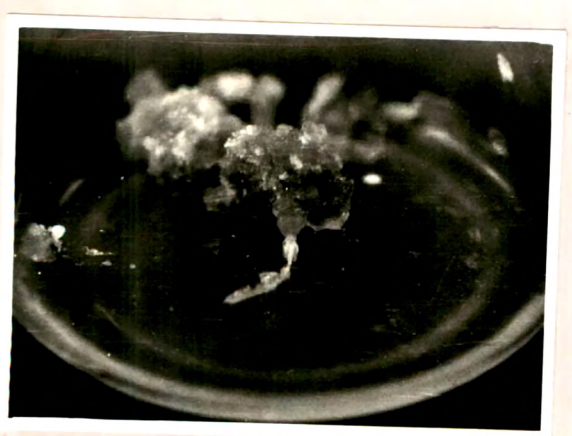


FIG. 34.

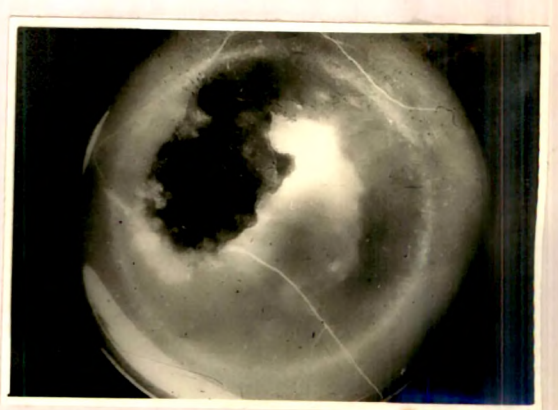


FIG. 35.

Table : 11. IAA effect, at different sucrose levels, on morphogenesis in diploid callus tissues of Nicotiana tabacum L.

Medium	IAA (mg/l)	Sucrose (%)	Morphogenic response	Frequency response (%)	Time taken for response
MS	-	2.0	Shoot	++	Many shoots in 4 weeks
"	0.175	2.0	-	-	-
"	0.175	3.0	Flower bud like structures	+	4 weeks
"	0.25	2.0	-	-	-
"	0.25	3.0	-	-	-
"	0.30	1.0	-	-	-
"	0.30	2.0	Shoot	++	Solitary shoots in 4 weeks
"	0.30	3.0	Shoot	++	Solitary shoot on Day 12
"	0.30	6.0	Root	+	Day 12
"	0.50	2.0	Roots	+	4 weeks
"	2.0	1.0	-	-	-
"	2.0	2.0	Roots	+++	4 weeks
"	2.0	3.0	Roots	+++	13 - 15 days
"	2.0	6.0	Roots	+	Day 12
"	3.0	1.0-6.0	-	-	-
"	4.0	1.0-6.0	-	-	-

Frequency response :

-	Nil
+	20-25 %
++	40-50 %
+++	60-75 %
++++	85-100%

Fig. 36. Root differentiation from diploid callus on MS + 2.0 mg/l IAA + 2% sucrose (4 weeks).

Fig. 37. Root differentiation from diploid callus on MS + 2.0 mg/l IAA + 3% sucrose (13-15 days).

Fig. 38. Root differentiation from diploid callus on MS + 2.0 mg/l IAA + 6% sucrose (12 days).

Fig. 39. Root differentiation from diploid callus on MS + 10 mg/l GA_3 + 2% sucrose (5 weeks).

Fig. 40. Shoot differentiation from diploid callus on MS + 0.03 mg/l IAA + 1.0 mg/l KN + 2% sucrose (3 weeks).

Fig. 41. Shoot and root differentiation from diploid callus on MS + 0.25 mg/l IAA + 0.175 mg/l KN + 3% sucrose (2 weeks).

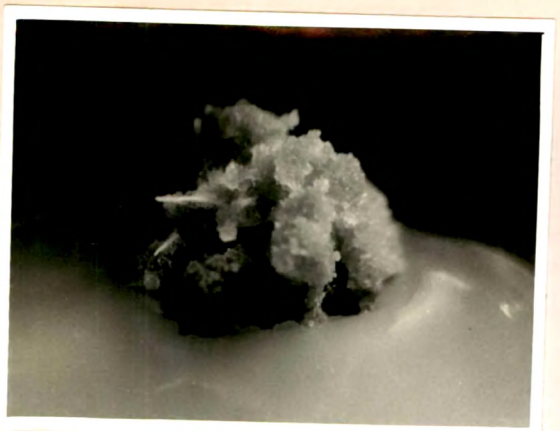


FIG. 36.

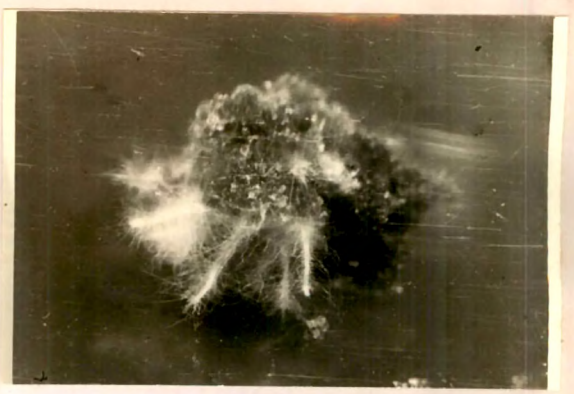


FIG. 37.

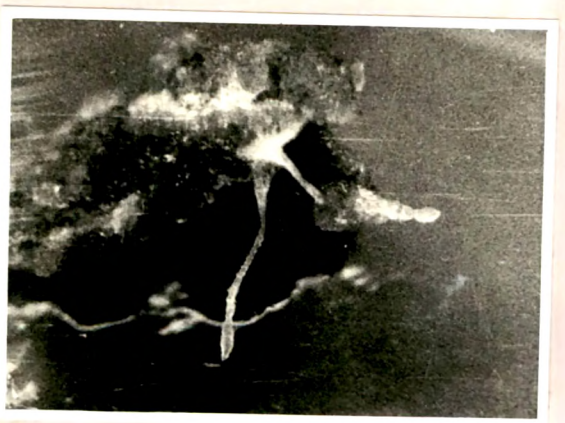


FIG. 38.

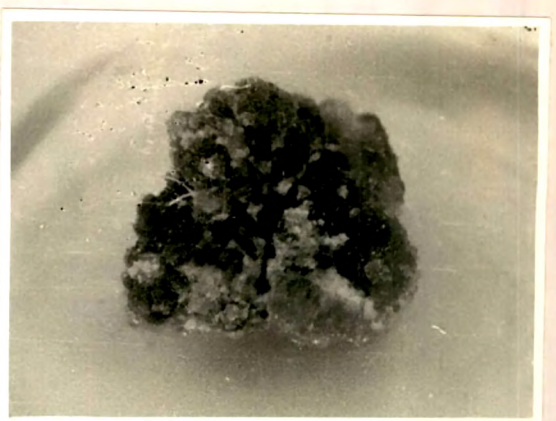


FIG. 39.

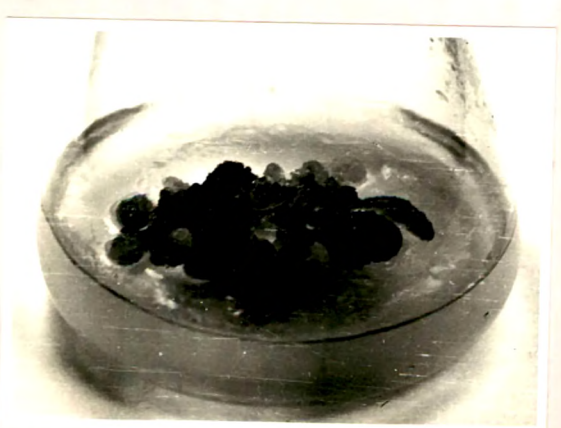


FIG. 40.

FIG. 41.

IAA and 1% sucrose. Roots were, however, differentiated when in the same medium sucrose level was increased to 2%. Roots were differentiated during the fourth week of culture with 75% frequency (Fig. 36, Table 11). In the same medium further increase of sucrose level to 3% also induced root differentiation during the second week of culture with about 75% frequency (Fig. 37, Table 11). Though further increase of sucrose level to 6% in the same medium resulted in root differentiation during the second week of culture, the frequency of response dropped to a mere 25% (Fig. 38, Table 11).

MS medium supplemented with 3.0 and 4.0 mg/l IAA and sucrose (1-6%) did not invoke any morphogenic response from diploid tobacco callus tissues.

Expt. 13. Influence of GA_3 on organogenesis

To study the influence of GA_3 on differentiation in callus cultures of floral bud origin, it was incorporated alone into MS basal medium in a wide range of concentrations as shown below :

i) MS + 5.0 mg/l GA_3 + 2% sucrose
ii) " + 10.0 mg/l GA_3 + "
iii) " + 15.0 mg/l GA_3 + "
iv) " + 20.0 mg/l GA_3 + "

v) MS + 25 mg/l GA_3 + 2% sucrose

vi) " + 50 mg/l GA_3 + 2% sucrose

vii)" + 100 mg/l GA_3 + 2% sucrose

Of all the concentrations of GA_3 tested, the only one to be effective was 10.0 mg/l. At that particular level of GA_3 roots were differentiated during the fifth week of culture with 10% frequency. The roots were scanty, small and thin (Fig. 39, Table 12).

Expt. 14. Influence of other phytohormones and adenine sulfate on differentiation

Besides IAA and GA_3, other phytohormones like NAA, 2,4-D, IBA, KN and MAP were tried singly to induce organogenesis in diploid tobacco callus tissues. NAA, 2,4-D and IBA were incorporated into MS medium in a wide range of concentrations (0.1 - 4.0 mg/l). No organogenetic response was obtained with any of these auxins tested. KN (0.02 - 2.0 mg/l) alone also proved ineffective in invoking organogenesis. 6-dimethylaminopurine (MAP) at concentrations ranging from 0.4 - 2.0 mg/l too did not facilitate organ formation. Adenine sulfate (10 - 500 mg/l) also failed to induce organogenesis in diploid tobacco callus tissues.

Table : 12. GA_3 effect on morphogenesis in diploid callus tissues of Nicotiana tabacum L.

Medium	GA_3 (mg/l)	Sucrose (%)	Morphogenic response	Frequency Response (%)	Time taken for response
MS	5.0	2.0	-	-	-
"	10.0	2.0	Roots	10.0	5 weeks
"	15.0	2.0	-	-	-
"	20.0	2.0	-	-	-
"	25.0	2.0	-	-	-
"	50.0	2.0	-	-	-
"	100.0	2.0	-	-	-

Frequency response : - Nil

Expt. 15. Influence of IAA and kinetin interactions on organogenesis

To study the influence of IAA and KN interactions on organogenesis in diploid tobacco callus tissues, they were incorporated into MS basal medium in a wide range of concentrations as given below :

i) MS + 0.03 mg/l IAA + 1.0 mg/l KN + 2% sucrose
ii) " + 0.25 mg/l IAA + 0.0175 mg/l KN + 3% sucrose
iii) " + " + 0.175 mg/l KN + "
iv) " + 0.35 mg/l IAA + 0.035 mg/l KN + "
v) " + " + 0.35 mg/l KN + "
vi) " + 0.5 mg/l IAA + 0.05 mg/l KN + "
vii) " + " + 0.5 mg/l KN + "
viii) " + 0.75 mg/l IAA + 0.075 mg/l KN + "
ix) " + " + 0.75 mg/l KN + "
x) " + 1.0 mg/l IAA + 0.1 mg/l KN + "
xi) " + " + 1.0 mg/l KN + "
xii) " + 2.0 mg/l IAA + 0.02 mg/l KN + 2% sucrose
xiii) " + " + 0.2 mg/l KN + 2% sucrose
xiv) " + " + 0.2 mg/l KN + 3% sucrose
xv) " + " + 0.4 mg/l KN + 3% sucrose
xvi) " + " + 0.5 mg/l KN + 2% sucrose
xvii) " + " + 1.0 mg/l KN + 2% sucrose
xviii) " + " + 2.0 mg/l KN + 2% sucrose

xix) MS + 2.0 mg/l IAA + 2.0 mg/l KN + 3% sucrose

xx) " + 4.0 mg/l IAA + 0.4 mg/l KN + 2% sucrose

xxi) " + 4.0 mg/l IAA + 0.4 mg/l KN + 3% sucrose

xxii) " + " + 4.0 mg/l KN + 2% sucrose

xxiii) " + " + " + 3% sucrose

On MS medium containing 0.03 mg/l IAA, 1.0 mg/l KN and 2% sucrose, shoots were differentiated during the third week of culture with 40-50% frequency (Fig. 40, Table 13).

No organogenetic response was obtained from diploid tobacco callus tissues cultured on 3% sucrose containing medium supplemented with IAA (0.25 mg/l) and KN (0.0175 mg/l). In the same medium, however, the increase of KN level to 0.175 mg/l resulted in the differentiation of leafy shoots with roots during the second week of culture with 50-60% frequency (Fig. 41, Table 13).

Increase of IAA concentration from 0.25 mg/l to 0.35 mg/l, in presence of 0.035 mg/l KN in 3% sucrose containing medium did not invoke any organogenic response. However, shoots with roots were differentiated on increasing KN level to 0.35 mg/l in the same medium. The shoots appeared during the second week of culture with 35-50% frequency (Fig. 42, Table 13).

Similarly, no organogenetic response was obtained from

Fig. 42. Shoot and root differentiation from diploid callus on MS + 0.35 mg/l IAA + 0.35 mg/l KN + 3% sucrose (2 weeks).

Fig. 43. Shoot and root differentiation from diploid callus on MS + 0.5 mg/l IAA + 0.5 mg/l KN + 3% sucrose (2 weeks).

Fig. 44. Shoot differentiation from diploid callus on MS + 0.75 mg/l IAA + 0.75 mg/l KN + 3% sucrose (2 weeks); note clumping of shoot.

Fig. 45. Shoot differentiation from diploid callus on MS + 1.0 mg/l IAA + 1.0 mg/l IAA + 3% sucrose (2 weeks) ; note clumping and stunted growth of shoots.

Fig. 46. Shoot differentiation from diploid callus on MS + 2.0 mg/l IAA + 0.2 mg/l KN + 3% sucrose (2 weeks).

Fig. 47. Shoot differentiation from diploid callus on MS + 2.0 mg/l IAA + 0.5 mg/l IN + 2% sucrose (4 weeks).

FIG. 42.

FIG. 43.

FIG. 44.

FIG. 45.

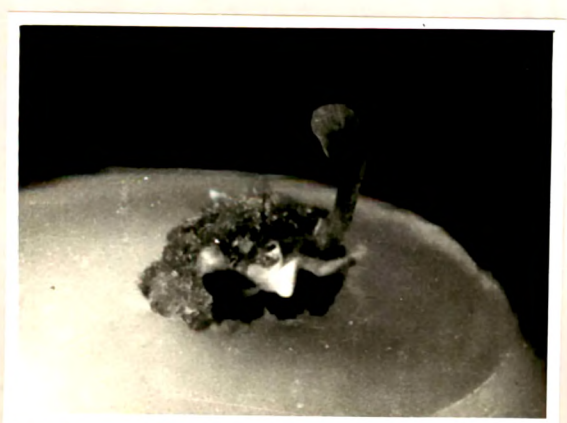


FIG. 46.

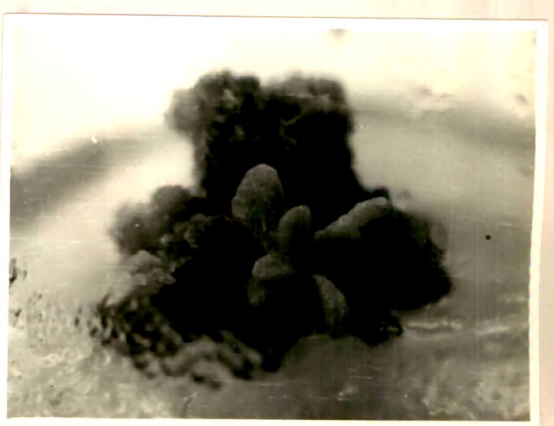


FIG. 47.

diploid tobacco callus tissues cultured on 3% sucrose containing medium supplemented with 0.5 mg/l IAA and 0.05 mg/l KN. On the other hand, shoots with roots were differentiated when the KN level was increased to 0.5 mg/l in the above medium. The organ differentiation occurred during the second week of culture with 80-90% frequency (Fig. 43, Table 13).

Similar pattern was observed with incorporation of high IAA (0.75 mg/l) in presence of low KN (0.075 mg/l). However, the increase of KN concentration to 0.75 mg/l in the same medium resulted in the differentiation of shoots during the second week of culture with about 75% frequency. The differentiated shoots had tendency to remain aggregated and were stunted (Fig. 44, Table 13).

No morphogenetic response was likewise registered in diploid tobacco callus tissues cultured on 3% sucrose containing MS medium supplemented with 1.0 mg/l IAA and 0.1 mg/l KN. However, the increase of KN level to 1.0 mg/l in the above medium resulted in differentiation of shoots with about 40-50% frequency during the second week of culture (Fig. 45, Table 13). The shoots were stunted and remained clustered.

Using high IAA (2.0 mg/l) in combination with KN

(0.02 and 0.2 mg/l) in MS medium containing 2% sucrose did not induce organogenesis in callus tissues. Shoots were differentiated, however, when sucrose level was raised to 3% in MS medium supplemented with 2.0 mg/l IAA and 0.2 mg/l KN. The shoots differentiated with about 70-80% frequency during the second week of culture (Fig. 46, Table 13).

Shoots were differentiated from callus tissues when KN concentration was increased from 0.2 to 0.5 mg/l, in presence of 2.0 mg/l IAA and 2% sucrose. The shoots were solitary and did not grow beyond four leaf stage. The shoots were differentiated during the fourth week of culture with about 25% frequency (Fig. 47, Table 13).

No organogenesis was observed from the callus tissues cultured on 2% sucrose containing medium supplemented with 2.0 mg/l IAA and 0.4, 1.0 or 2.0 mg/l KN. Shoots were, however, differentiated on 3% sucrose containing MS medium supplemented with 2.0 mg/l IAA and 2.0 mg/l KN. The shoots were dwarf and remained clumped. The caulogenic response occurred during the second week of culture with 40-50% frequency (Fig. 48, Table 13).

Increasing the concentration of IAA still higher (4.0 mg/l) in the MS medium supplemented with 0.4 or 4.0 mg/l KN in

Table : 13. Influence of IAA and Kinetin interactions on morphogenesis in diploid callus cultures of Nicotiana tabacum L.

Medium	IAA (mg/l)	KN (mg/l)	Sucrose (%)	Morphogenic response	Frequency response (%)	Time taken for response
MS(Basal)	0.03	1.0	2.0	Shoots	++	3 weeks
"	0.25	0.0175	3.0	-	-	-
"	0.25	0.175	3.0	Shoots without roots	++	2 weeks
"	0.35	0.035	3.0	-	-	-
"	0.35	0.35	3.0	Shoots with roots	++	2 weeks
"	0.5	0.05	3.0	-	-	-
"	0.5	0.5	3.0	Shoots with roots	++++	2 weeks
"	0.75	0.075	3.0	-	-	-
"	0.75	0.75	3.0	Shoots	+++	2 weeks
"	1.0	0.1	3.0	-	-	-
"	1.0	1.0	3.0	Shoots	++	2 weeks
"	2.0	0.02	2.0	-	-	-
"	2.0	0.2	2.0	-	-	-
"	2.0	0.2	3.0	Shoots	+++	2 weeks
"	2.0	0.4	2.0	-	-	-
"	2.0	0.5	2.0	Shoots	+	4 weeks
"	2.0	1.0	2.0	-	-	-
"	2.0	2.0	2.0	-	-	-
"	2.0	2.0	3.0	Shoots	++	2 weeks
"	4.0	0.4	2.0	-	-	-
"	4.0	0.4	3.0	-	-	-
"	4.0	4.0	2.0	-	-	-
"	4.0	4.0	3.0	-	-	-

Frequency response :

-	Nil
+	20-25 %
++	40-50 %
+++	60-75 %
+++	85-100%

Fig. 48. Shoot differentiation from diploid callus on MS + 2.0 mg/l IAA + 2.0 mg/l KN + 3% sucrose (2 weeks); noted clumping of shoots and stunted growth.

Fig. 49. Shoot differentiation from root explant of diploid tobacco plant on MS + 2.0 mg/l IAA + 0.4 mg/l KN + 2% sucrose (4 weeks).

Fig. 50. Shoot differentiation from shoot explant of diploid tobacco plant on MS + 2.0 mg/l IAA + 0.4 mg/l KN + 2% sucrose (3 weeks); shoot differentiation occurred subsequent of callusing from stem explant.

Fig. 51. Shoot differentiation from the leaf explant of diploid tobacco plant on MS + 2.0 mg/l IAA + 0.4 mg/l KN + 2% sucrose (4 weeks).

FIG. 48.

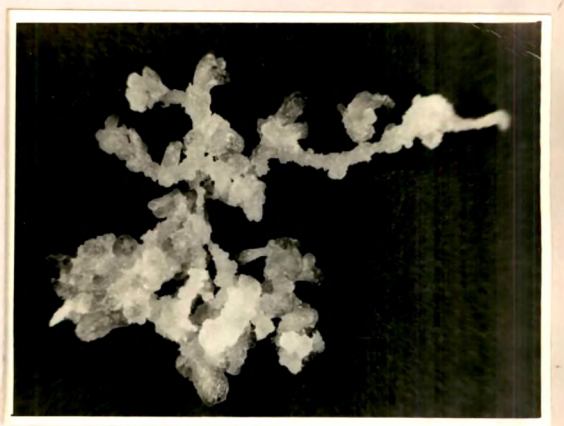


FIG. 49.

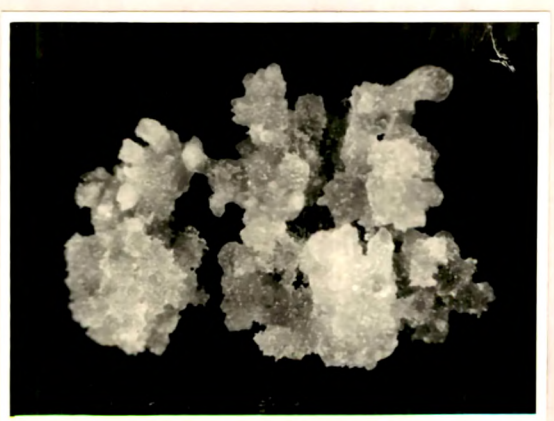


FIG. 50.

FIG. 51.

presence of 2 or 3% sucrose did not bring about differentiation.

Expt. 16. Influence of added phenolic acids on organogenesis

In order to study the influence of phenolic acids on organogenesis, out of the whole array of media whereon differentiation occurred, only three media were chosen. These media were characterized by consistency, rapidity and high frequency of response. The three media were :

(a) MS basal + 2.0 mg/l IAA + 3% sucrose (root differentiating medium),

(b) MS basal + 0.3 mg/l IAA + 3% sucrose (shoot differentiating medium) and

(c) MS basal + 0.5 mg/l IAA + 0.5 mg/l KN + 3% sucrose (shoot differentiating medium).

In the above mentioned media t-cinnamic acid (100, 500 μM), Caffeic acid (100, 500 μM), Ferulic acid (100, 500 μM) and p-Hydroxybenzoic acid (1, 10 μM) were added separately. The addition of these phenolic acids into the above mentioned media brought about complete inhibition of organ differentiation.

Expt. 17. Organogenesis in root, stem and leaf explants of diploid origin

The root,stem and leaf explants of plantlets differentiated

from diploid callus on MS basal medium were used to study their organogenetic capacities. The explants were excised aseptically and planted on MS medium supplemented with 2.0 mg/l IAA, 0.4 mg/l KN and 2.0 per cent sucrose.

The root explants grew in length and were covered with small amounts of callus and then the differentiation of leafy shoots followed in the fourth week of culture. The shoots were more or less leaf like structures and had tendency to stick together (Fig. 49).

The stem explants callus profusely and later by the end of third week in culture leaf like structures differentiated from the callus masses (Fig. 50).

The leaf explants by the third week grew three to four times their size at the time of inoculation and assumed a mottled appearance. It is during the fourth week that many shoots emerge from the base of the leaf in a bunch (Fig. 51). No callus formation was observed.

Section C-II. Organogenesis in Haploid Callus Cultures of *Nicotiana tabacum* L.

C - II. Organogenesis in haploid callus cultures of Nicotiana tabacum L.

Callus cultures derived from the shoot explants of haploid plantlets and maintained on MS medium supplemented with 2.0 mg/l each of IAA, NAA and KN, were later transferred to MS medium containing varying levels of phytohormones, singly and in combinations, and sucrose to evoke morphogenetic responses.

Expt. 18. Influence of IAA on organogenesis

Healthy looking, green callus masses were cultured on MS medium supplemented with a wide range of IAA (0.175 - 4.0 mg/l) and sucrose (1 - 6%) as shown below :

i) MS + 2% sucrose
ii) MS + 0.175 mg/l IAA + 2% sucrose
iii) " + 0.175 mg/l IAA + 3% sucrose
iv) " + 0.25 mg/l IAA + 2% sucrose
v) " + " + 3% sucrose
vi) " + 0.3 mg/l IAA + 1% sucrose
vii) " + 0.3 mg/l IAA + 2% sucrose
viii) " + 0.3 mg/l IAA + 3% sucrose
ix) " + 0.3 mg/l IAA + 6% sucrose
x) " + 0.5 mg/l IAA + 2% sucrose

xi) MS + 2.0 mg/l IAA + 1% sucrose

xii) " + " + 2% sucrose

xiii) " + " + 3% sucrose

xiv) " + " + 6% sucrose

xv) " + 3.0 mg/l IAA + 1% sucrose

xvi) " + " + 2% sucrose

xvii) " + " + 3% sucrose

xviii) " + " + 6% sucrose

xix) " + 4.0 mg/l IAA + 1% sucrose

xx) " + " + 2% sucrose

xxi) " + " + 3% sucrose

xxii) " + " + 6% sucrose

The cultures were incubated at 26±2° in continuous light and periodic observations were made. The results are presented in Table 14 and Figures 52-61.

Callus tissues cultured on MS basal medium containing only 2% sucrose served as the control. On this medium shoots were differentiated during the third week of culture with about 50% frequency. The shoots differentiated numbered 3-8 per callus mass (Fig. 52, Table 14).

On MS medium containing 0.175 mg/l IAA and 2% sucrose, no organogenetic response was obtained even after four weeks of culture. In the same medium the increase of sucrose level

to 3%, however, resulted in the differentiation of shoots with about 20-25% frequency during the third week of culture (Fig. 53, Table 14).

On the other hand, 0.25 mg/l IAA in combination with 2 or 3% sucrose failed to invoke either shoot or root differentiation.

On MS medium containing 0.3 mg/l IAA and 1% sucrose similarly, no organogenic response was observed. However, with the increase of sucrose level to 2% at the same auxin concentration, leaf like appendages differentiated which failed to grow further. These leaf like structures were obtained latest by day 15 in culture and with 30-40% frequency (Fig. 54, Table 14). Further increase of sucrose to 3% in the same medium resulted in the differentiation of a well defined solitary shoot. Such shoot differentiation occurred by day 9 of culture with 75-80% frequency (Fig. 55, Table 14). When 6% sucrose was used in the same IAA (0.3 mg/l) containing medium, there was shift in organogenic response from shoot to root formation. No more than 3-4 roots were differentiated per callus mass. The differentiation of roots occurred during the second week of culture with 40-50% frequency (Fig. 56, Table 14).

On MS medium containing 0.5 mg/l IAA and 2% sucrose, one

Fig. 52. Shoot differentiation of haploid callus of *N. tabacum* on MS basal + 2% sucrose (5 weeks).

Fig. 53. Shoot differentiation from haploid callus on MS + 0.175 mg/l IAA + 3% sucrose (3 weeks).

Fig. 54. Shoot differentiation from haploid callus on MS + 0.3 mg/l IAA + 2% sucrose (15 days).

Fig. 55. Very well developed shoot differentiated from haploid callus on MS + 0.3 mg/l IAA + 3% sucrose (9 days for differentiation).

Fig. 56. Root differentiation from haploid callus on MS + 0.3 mg/l IAA + 6% sucrose (12 days).

Fig. 57. Root differentiation from haploid callus on MS + 0.5 mg/l IAA + 2% sucrose (15 days).

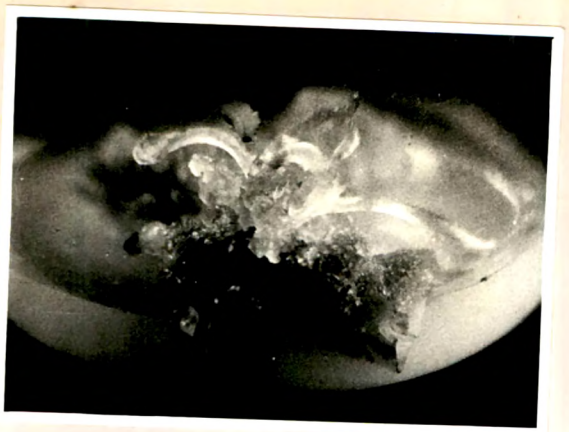


FIG. 52.

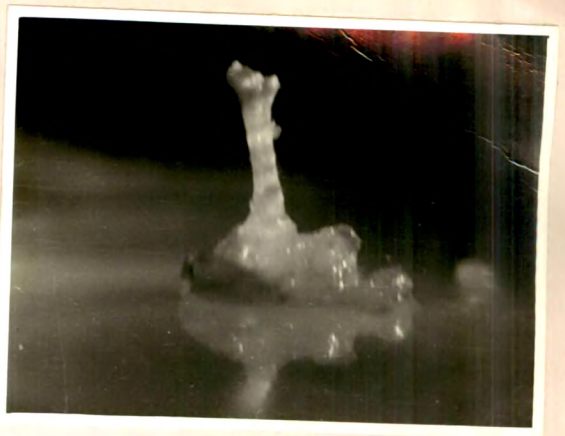


FIG. 53.

FIG. 54.

FIG. 55.

FIG. 56.

FIG. 57.

Table : 14. Influence of IAA at different sucrose levels on organogenesis in haploid callus tissues of *N. tabacum* var. Anand-2.

Culture conditions : The cultures were incubated at 26±2° in continuous light.

IAA (mg/l)	Sucrose (%)	Morphogenetic response	Frequency response (%)	Time taken for response
-	2.0	Shoots	++	15 days
0.175	2.0	-	-	-
0.175	3.0	Shoots	+	21 days
0.3	1.0	-	-	-
0.3	2.0	Shoot	++	15 days
0.3	3.0	Shoot	+++	9 days
0.3	6.0	Roots	+	12 days
0.5	2.0	Roots	+	15 days
2.0	1.0	Roots	+	15 days
2.0	2.0	Roots	+	15 days
2.0	3.0	Roots	+++	9 days
2.0	6.0	Roots	++	12 days
3.0	1.0-6.0	-	-	-
4.0	1.0-6.0	-	-	-

Frequency response : - = No response
\+ = 15-25%
++ = 40-60%
+++ = 60-80%
++++ = 80-100%

Fig. 58. Root differentiation from haploid callus on MS + 2.0 mg/l IAA + 1% sucrose (15 days).

Fig. 59. Root differentiation from haploid callus on MS + 2.0 mg/l IAA + 2% sucrose (15 days).

Fig. 60. Root differentiation from haploid callus on MS + 2.0 mg/l IAA + 3% sucrose (9 days).

Fig. 61. Root differentiation from haploid callus on MS + 2.0 mg/l IAA + 6% sucrose (9 days).

Fig. 62. Shoot differentiation from haploid callus on MS + 0.03 mg/l IAA + 1.0 mg/l KN * + 2% sucrose (21 days).

Fig. 63. Shoot and root differentiation from haploid callus on MS + 0.25 mg/l IAA + 0.175 mg/l KN + 3% sucrose (15 days).

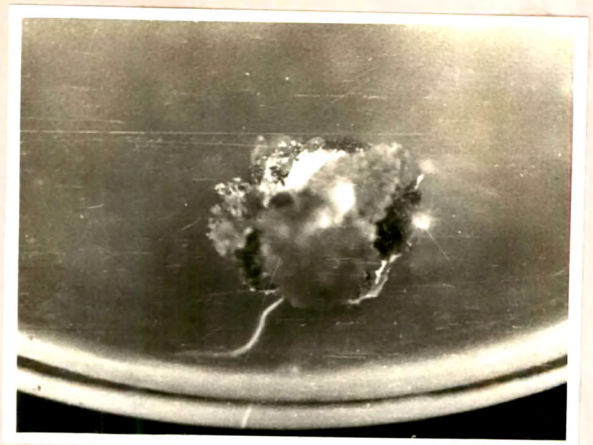


FIG. 58.

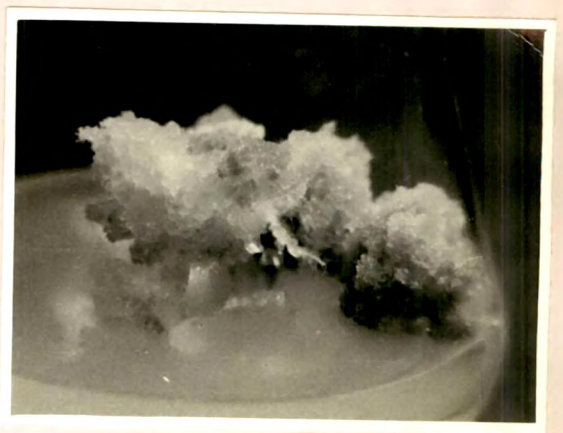


FIG. 59.

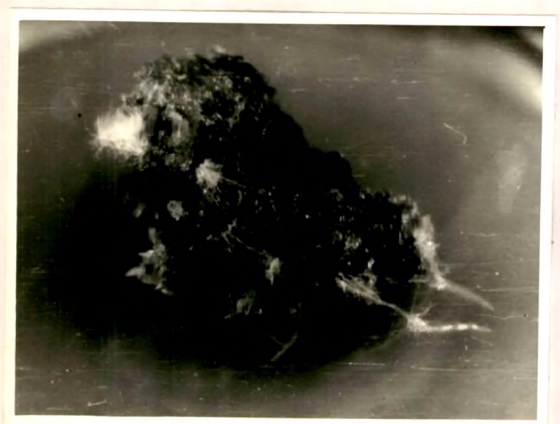


FIG. 60.

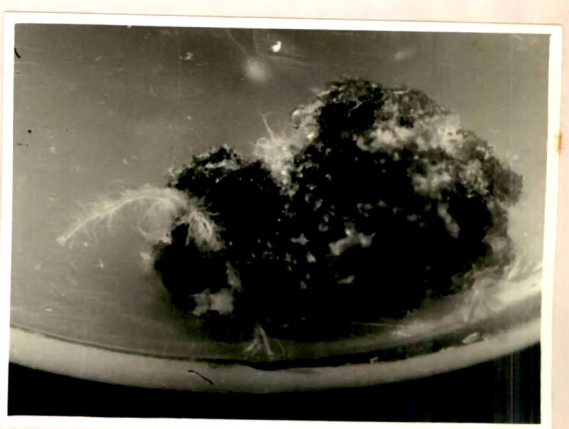


FIG. 61.

FIG. 62.

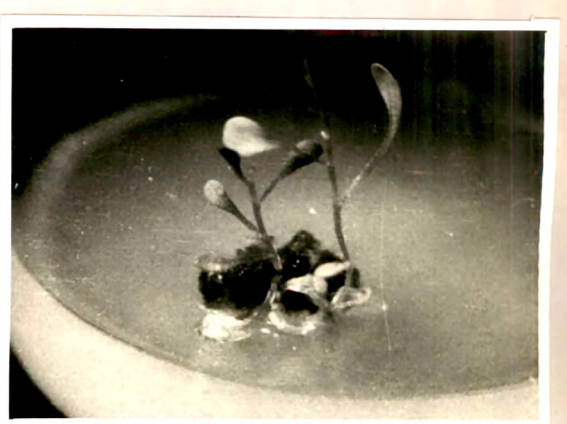


FIG. 63.

or two roots were differentiated from haploid tobacco callus cultures. The roots usually appeared during the third week of culture with about 30% frequency (Fig. 57, Table 14).

Higher concentration of IAA (2.0 mg/l) induced root formation on a wide range of sucrose level (1 - 6 %). With 1 and 2% sucrose in the medium, 2-4 roots differentiated by day 15 of culture with 15-20% frequency (Figs. 58, 59; Table 14). With the increase of sucrose level to 3%, the number of roots differentiated per callus mass increased to 8-10. The time taken for differentiation was 9 days and the frequency of response 60-80% (Fig. 60, Table 14). With further increase of sucrose to 6% in the medium, 6-7 roots were differentiated per callus mass by day 12 of culture with 40-50% frequency (Fig. 61, Table 14).

MS medium supplemented with 3.0 and 4.0 mg/l IAA and sucrose 1-6%) did not invoke any morphogenic response from haploid tobacco callus tissues even after 4 weeks of culture.

Expt. 19. Influence of other phytohormones and adenine sulfate on organogenesis

Besides IAA, other phytohormones like NAA, 2,4-D, IBA, KN, MAP and GA_3 were tried singly to induce organogenesis in haploid tobacco callus tissues. NAA, IBA and 2,4-D were

incorporated into MS basal medium in a wide range of concentrations (0.1 - 4.0 mg/l). No organogenetic response was obtained by any of these auxins tested. KN (0.02 - 2.0 mg/l) alone also proved ineffective in invoking morphogenesis. 6-dimethylamino purine (MAP) at concentrations ranging from 0.4 - 2.0 mg/l too did not induce organ formation. GA_3 (5-100 mg/l) and Adenine sulfate (10-500 mg/l) also proved ineffective in inducing organogenesis in haploid tobacco callus tissues.

Expt. 20. Influence of IAA and kinetin interactions on Organogenesis

To study the influence of IAA and KN interactions on organogenesis in haploid tobacco callus tissues, they were incorporated into MS basal medium in a wide range of concentrations as given below :

i) MS + 0.03 mg/l IAA + 1.0 mg/l KN + 2% sucrose

ii) " + 0.25 mg/l IAA + 0.0175 mg/l KN + 3% sucrose

iii) " + " + 0.175 mg/l KN + "

iv) " + 0.35 mg/l IAA + 0.035 mg/l KN + "

v) " + " + 0.35 mg/l KN + "

vi) " + 0.5 mg/l IAA + 0.05 mg/l KN + "

vii) " + " + 0.5 mg/l KN + "

viii) " + 0.75 mg/l IAA + 0.075 mg/l KN + "

ix) MS + 0.75 mg/l IAA + 0.75 mg/l KN + 3% sucrose

x) " + 1.0 mg/l IAA + 0.1 mg/l KN + 3% sucrose

xi) " + " + 1.0 mg/l KN + "

xii) " + 2.0 mg/l IAA + 0.02 mg/l KN + 2% sucrose

xiii) " + " + 0.2 mg/l KN + "

xiv) " + " + " + 3% sucrose

xv) " + " + 0.4 mg/l KN + "

xvi) " + " + 0.5 mg/l KN + 2% sucrose

xvii) " + " + 1.0 mg/l KN + "

xviii) " + " + 2.0 mg/l KN + "

xix) " + " + " + 3% sucrose

xx) " + 4.0 mg/l IAA + 0.4 mg/l KN + 2% sucrose

xxi) " + " + " + 3% sucrose

xxii) " + " + 4.0 mg/l KN + 2% sucrose

xxiii) " + " + " + 3% sucrose

On MS medium containing 0.03 mg/l IAA, 1.0 mg/l KN and 2% sucrose, differentiation of shoots occurred during the third week of culture with about 50% frequency. Usually 4-8 shoots were differentiated per callus mass (Fig. 62, Table 15).

No organogenetic response was obtained from haploid tobacco callus tissues cultured on 3% sucrose containing medium supplemented with IAA (0.25 mg/l) and KN (0.0175 mg/l). In the same medium, however, the increase of KN level to

0.175 mg/l resulted in the differentiation of very well defined shoots during the second week of culture with 50-60% response. Per callus mass 2-3 shoots were differentiated (Fig. 63, Table 15).

Increase of IAA concentration from 0.25 mg/l to 0.35 mg/l, in presence of 0.035 mg/l KN in 3% sucrose containing medium did not evoke any organogenetic response. However, solitary shoots with roots were differentiated on increasing KN level to 0.35 mg/l in the same medium. The shoots appeared during the second week of culture with 50-60% frequency (Fig. 64, Table 15).

Similarly no organogenetic response was obtained from haploid tobacco callus tissues cultured on 3% sucrose containing MS medium supplemented with 0.5 mg/l IAA and 0.05 mg/l KN. On the other hand, 2-4 shoots were differentiated per callus mass when the KN level was increased to 0.5 mg/l in the above medium. The shoots were differentiated during the second week of culture with 80-90% frequency (Fig. 65, Table 15).

Same pattern was observed with incorporation of high IAA (0.75 mg/l) in presence of low KN (0.075 mg/l). However, with the increase of KN concentration to 0.75 mg/l in the same medium, solitary shoot with roots were differentiated

in the second week of culture with 30-40% frequency (Fig. 66, Table 15).

No morphogenetic response was likewise registered in haploid tobacco callus tissues cultured on 3% sucrose containing MS medium supplemented with 1.0 mg/l IAA and 0.1 mg/l KN. However, the increase of KN level to 1.0 mg/l in the above medium resulted in the differentiation of shoots with 30-40% frequency during the second week of culture (Fig. 67, Table 15).

Using high IAA (2.0 mg/l) in combination with KN (0.02 and 0.2 mg/l) in MS medium containing 2% sucrose did not induce organogenesis in the callus tissues. Shoots were differentiated, however, when sucrose level was raised to 3% in MS medium supplemented with 2.0 mg/l IAA and 0.2 mg/l KN. The shoots differentiated with reduced (20-30%) frequency during the second week of culture (Fig. 68, Table 15).

Very well defined shoots were differentiated from the callus tissues when KN concentration was increased from 0.2 to 0.4 mg/l in presence of 2.0 mg/l IAA and 3% sucrose. The frequency of response was 30-40% and shoot were differentiated during third week of culture (Fig. 69, Table 15).

No organogenesis was observed from the callus tissues

Fig. 64. Shoot and root differentiation from haploid callus on MS + 0.35 mg/l IAA + 0.35 mg/l KN + 3% sucrose (15 days).

Fig. 65. Shoot differentiation from haploid callus on MS + 0.5 mg/l IAA + 0.5 mg/l KN + 3% sucrose (15 days).

Fig. 66. Shoot and root differentiation from haploid callus dn MS + 0.75 mg/l IAA + 0.75 mg/l KN + 3% sucrose (15 days).

Fig. 67. Shoot differentiation from haploid callus on MS + 1.0 mg/l IAA + 1.0 mg/l KN + 3% sucrose (15 days).

Fig. 68. Shoot differentiation from haploid callus on MS + 2.0 mg/l IAA + 0.2 mg/l KN + 3% sucrose (15 days).

Fig. 69. Shoot differentiation from haploid callus on MS + 2.0 mg/l IAA + 0.4 mg/l KN + 3% sucrose (21 days).

FIG. 64.

FIG. 65.

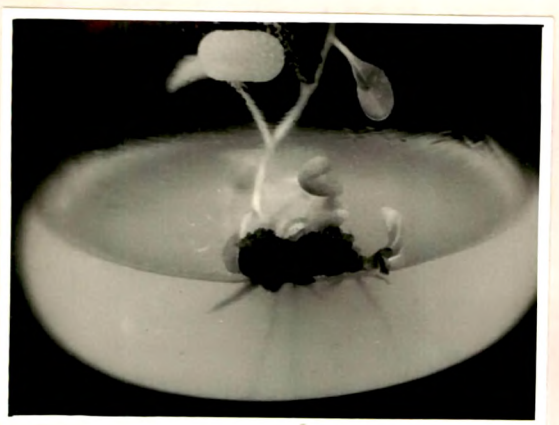


FIG. 66.

FIG. 67.

FIG. 68.

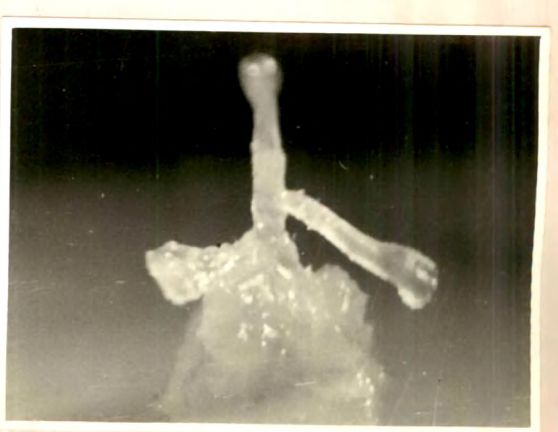


FIG. 69.

Table : 15. Influence of IAA and KN interactions on organogenesis in haploid callus tissues of N. tabacum L.

Note: All cultures were incubated at 26±2° in continuous light. Additions of IAA, KN and sucrose were made into MS (1962) basal medium.

IAA (mg/l)	KN (mg/l)	Sucrose (%)	Morphogenetic response	Frequency response (%)	Time taken(days)
0.03	1.0	2.0	Shoots	++	21
0.25	0.0175	3.0	-	-	-
0.25	0.175	3.0	Shoots & roots	++	15
0.35	0.035	3.0	-	-	-
0.35	0.35	3.0	Shoots & roots	++	15
0.5	0.05	3.0	-	-	-
0.5	0.5	3.0	Shoots	++++	15
0.75	0.075	3.0	-	-	-
0.75	0.75	3.0	Shoots & roots	++	15
1.0	0.1	3.0	-	-	-
1.0	1.0	3.0	Shoots	++	15
2.0	0.02	2.0	-	-	-
2.0	0.2	2.0	-	-	-
2.0	0.2	3.0	Shoots	+	15
2.0	0.4	3.0	Shoots	++	21
2.0	0.5	2.0	-	-	-
2.0	1.0	2.0	-	-	-
2.0	2.0	2.0	-	-	-
2.0	2.0	3.0	Shoots	++	12
4.0	0.4	2.0	-	-	-
4.0	0.4	3.0	-	-	-
4.0	4.0	2.0	-	-	-
4.0	4.0	3.0	-	-	-

\- = No response
\+ = 15-25 % response
++ = 40-60 % "
+++ = 60-80 % "
++++ = 80-100% "

Fig. 70. Shoot differentiation from haploid callus cultured on MS + 2.0 mg/l IAA + 2.0 mg/l KN + 3% sucrose (12 days).

Fig. 71. Root differentiation from haploid callus cultured on MS + 0.3 mg/l IAA + 500 uM t-cinnamic acid + 3% sucrose (6 days).

Fig. 72. Root differentiation from haploid callus cultured on MS + 0.3 mg/l IAA + 100 uM caffeic acid + 3% sucrose (9 days).

Fig. 73. Root differentiation from haploid callus cultured on MS + 2.0 mg/l IAA + 100 uM t-cinnamic acid + 3% sucrose (6 days).

Fig. 74. Root differentiation from haploid callus cultured on MS + 2.0 mg/l IAA + 500 uM caffeic acid + 3% sucrose (6 days).

Fig. 75. Root differentiation from haploid callus cultured on MS + 0.5 mg/l IAA + 0.5 mg/l KN + 100 uM caffeic acid + 3% sucrose (6 days).

FIG. 70.

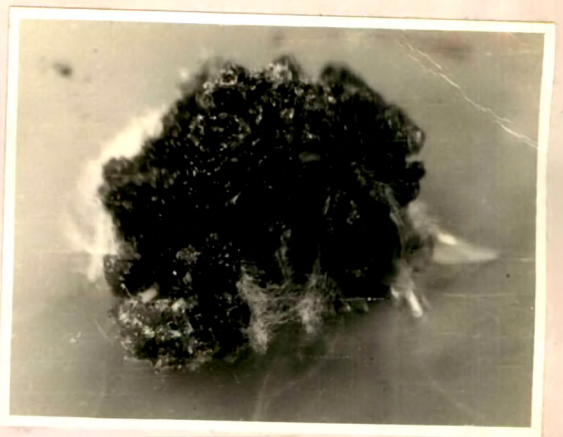


FIG. 71.

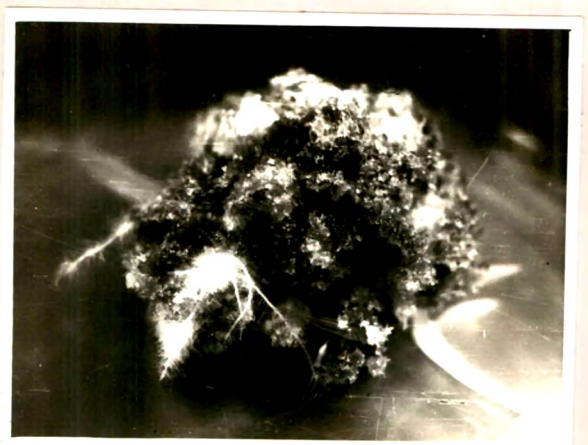


FIG. 72.

FIG. 73.

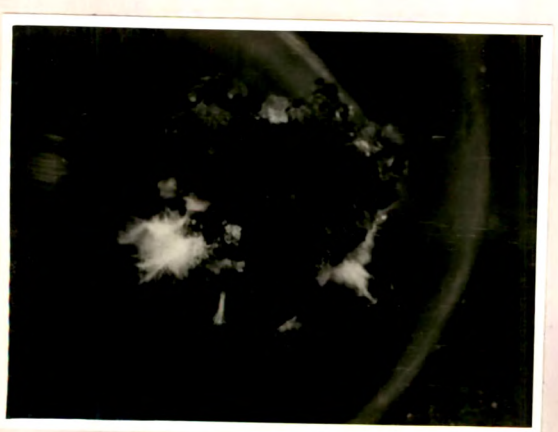


FIG. 74.

FIG. 75.

cultured on 2% sucrose containing MS medium supplemented with 2.0 mg/l IAA and 0.5, 1.0 or 2.0 mg/l KN. Shoot were, however, differentiated on 3% sucrose containing MS medium supplemented with 2.0 mg/l IAA and 2.0 mg/l KN. The shoots appeared during the second week of culture with 30-40% frequency (Fig. 70, Table 15).

Increasing the concentration of IAA still higher (4.0 mg/l) in MS medium supplemented with 0.4 or 4.0 mg/l KN in presence of 2 or 3% sucrose did not bring about any organ differentiation in haploid callus tissues.

Expt. 21. Influence of added phenolic acids on organogenesis

In order to study the influence of phenolic acids on organogenesis, out of the whole array of media whereon differentiation occurred, only three media were chosen. These media were characterised by consistency, rapidity and high frequency of response. The three media were :

(a) MS basal + 2.0 mg/l IAA + 3% sucrose (root differentiating medium),

(b) MS basal + 0.3 mg/l IAA + 3% sucrose (shoot differentiating medium) and

(c) MS basal + 0.5 mg/l IAA + 0.5 mg/l KN + 3% sucrose (shoot differentiating medium).

In the above mentioned media t-Cinnamic acid (100, 500 μM), Caffeic acid (100, 500 μM), Ferulic acid (100, 500 μM) and p-Hydroxybenzoic acid (1, 10 μM) were added separately.

Addition of 500 μM t-cinnamic acid, 100 μM caffeic acid, 100 and 500 μM ferulic acid, and 1 and 10 μM p-hydroxybenzoic acid into MS medium containing 2.0 mg/l IAA, resulted in total inhibition of root differentiation. On incorporation of 100 μM t-cinnamic acid roots were differentiated with 60-80% frequency by day 6 in culture. (Fig. 71, Table 16). Roots were also differentiated on 500 μM caffeic acid containing medium with 50-60% frequency by day 6 of culture (Fig. 72, Table 16).

Incorporation of 100 μM t-cinnamic acid, 500 μM caffeic acid, 100 and 500 μM ferulic acid, and 1.0 and 10 μM p-hydroxybenzoic acid into MS medium containing 0.3 mg/l IAA resulted in complete inhibition of shoot differentiation. With the use of 500 μM t-cinnamic acid and 100 μM caffeic acid in the medium there was a shift in the organogenetic expression from shoot to root development (Figs. 73, 74; Table 16). In presence of 500 μM t-cinnamic acid in the medium, roots originated on day 6 and with 100 μM caffeic acid in the medium on day 9. The frequency of response in both the cases was 50-60%.

In MS medium containing 0.5 mg/l IAA and 0.5 mg/l KN,

Table : 16. Influence of added phenolic acids on organogenesis in haploid callus tissues of N. tabacum L.

Note : All cultures were incubated at 26±2° in continuous light. Additions of IAA, KN and phenolic acids were made into MS (1962) basal medium containing 3% sucrose.

IAA (mg/l)	KN (mg/l)	t-Cinnamic acid (uM)	Caffeic acid (uM)	Ferulic acid (uM)	p-HBA (uM)	Morphogenetic response	Frequency of response (%)	Time taken (days)
0.3	-	100	-	-	-	-	-	-
0.3	-	500	-	-	-	Roots	++	6
0.3	-	-	100	-	-	Roots	++	9
0.3	-	-	500	-	-	-	-	-
0.3	-	-	-	100/500	-	-	-	-
0.3	-	-	-	-	1/10	-	-	-
2.0	-	100	-	-	-	Roots	+++	6
2.0	-	500	-	-	-	-	-	-
2.0	-	-	100	-	-	-	-	-
2.0	-	-	500	-	-	Roots	++	6
2.0	-	-	-	100/500	-	-	-	-
2.0	-	-	-	-	1/10	-	-	-
0.5	0.5	100/500	-	-	-	-	-	-
0.5	0.5	-	100	-	-	Roots	++	6
0.5	0.5	-	500	-	-	-	-	-
0.5	0.5	-	-	100/500	-	-	-	-
0.5	0.5	-	-	-	1/10	-	-	-

- = No response ; + = 15-25% response; ++ = 40-60% response
+++ = 60-80% response; ++++ = 80-100% response

the incorporation of 100 and 500 μM t-cinnamic acid, 500 μM caffeic acid, 100 and 500 μM ferulic acid, and 1.0 and 10 μM p-hydroxybenzoic acid resulted in the suppression of shoot differentiation. However, instead of shoots, roots differentiated in presence of 100 μM caffeic acid in the medium (Fig. 75, Table 16). The roots differentiated on day 6 with 50-60% frequency.

Expt. 22. Organogenesis in root, shoot and leaf explants of haploid tobacco plantlets

Root, stem and leaves were excised from haploid plantlets derived from the anthers of N. tabacum. The explants were cultured on MS medium supplemented with 2.0 mg/l IAA and 0.4 mg/l KN in presence of 2% sucrose.

The root explant grew in dimensions during the early phases of culture. Later during the fourth week of culture the entire root explant gave rise to leafy shoots without prior formation of callus (Fig. 76).

The stem explant thickened in culture and was later covered with callus. No differentiation was obtained even after 6 weeks in culture (Fig. 77).

The leaf explant increased many fold in size during the first three weeks of culture. During the fourth week, the lower half of the leaf explant rolled up lengthwise and then many shoots appeared from the base of the leaf (Fig. 78).

Fig. 76. Shoot differentiation from root explant of haploid plant on MS + 2.0 mg/l IAA + 0.4 mg/l KN + 2% sucrose (4 weeks).

Fig. 77. Callusing from shoot explant of haploid plant on MS + 2.0 mg/l IAA + 0.4 mg/l KN + 2% sucrose (4 weeks).

Fig. 78. Shoot differentiation from leaf explant of haploid plant on MS + 2.0 mg/l IAA + 0.4 mg/l KN + 2% sucrose (4 weeks).

FIG. 76.

FIG. 77.

FIG. 78

Section D : Physiological Studies with Peroxidase, IAA Oxidase, MDH, PAL, Peroxidase isoenzymes and Phenolics during Growth of Diploid and Haploid Callus Tissues of *N. tabacum* L.

In experiments carried out earlier on and described in Chapter III, Sections B-I and B-II, it became obvious that diploid and haploid tissues of tobacco retained their growth potentials when cultured *in vitro*. Growth responses of callus cultures rested largely on the exogenous supply of growth hormones, singly or in combination.

In an attempt to elucidate the role of phytohormones in growth and biochemical changes associated therewith, the medium which supported rapid and maximum growth of both diploid and haploid callus tissues was used :

MS (basal)	+ IAA	+ NAA	+ KN	+ Sucrose.
	(2.0 mg/l)	(2.0 mg/l)	(2.0 mg/l)	(2%)

The results obtained are presented under the following heads :

(i) Studies with floral buds of *N. tabacum* during callus initiation,

(ii) Studies with floral bud callus of *N. tabacum* during its growth on standard medium,

(iii) Studies with haploid callus of *N. tabacum* during its growth on standard medium.

Expt. 23. Studies with floral buds of N. tabacum during callus initiation.

Flower buds (6-8 mm) of Nicotiana tabacum var. Anand-2 were surface sterilized, cut lengthwise into two halves and cultured on 40 ml of MS basal medium supplemented with 2.0 mg/l each of IAA, NAA and KN, and 2% sucrose. Explants weighing 500±60 mg by fresh weight were cultured per culture flask. The culture vessels were incubated in continuous light at 26±2° for a period of 30 days. Every third day till day 21, a fixed number of 5 replicates was harvested and analysed for growth, enzymes : peroxidase, IAA Oxidase, MDH and PAL, isoperoxidase banding patterns and also for phenolic content. Final reading was taken after incubation for 30 days.

(a) Growth :

Growth of floral buds in culture was measured in terms of fresh and dry weights and is presented in Fig. 79 and Table 17.

Growth of flower buds in culture followed a typical "double sigmoid growth curve". Fresh weight increased linearly during day 0 and day 15. It was followed by a plateau during day 15 to day 18 which acted as an interlude between two growth phases. On day 18 small bits of callus appeared, originating from the tip and cut edges of sepals. The second phase of linear growth was during day 18 and day 30. During the entire

30 day period fresh weight increased 4.2 fold. On dry weight basis a distinct lag phase for initial three days was conspicuous. Thereafter, dry mass increased till day 15. During day 15 and day 18 it remained essentially stable. The second growth phase commenced from day 18 and lasted till day 30. Dry mass increased 3.6 fold during the 30 day culture period. The callus initiation was thus immediately preceded by a period of slow growth.

(b) Peroxidase :

Total and specific peroxidase activity of flower buds of tobacco in culture is illustrated in Fig. 79 and Table 17.

No peroxidase activity was detectable on day 0 of culture. Thereof, total peroxidase activity increased till day 6, attaining first peak value during the culture period. The activity declined during day 6 and day 9. Thereafter, till day 15 total peroxidase activity increased steadily reaching second peak value. The activity declined again, but rather marginally, during day 15 and day 18. From day 18 onwards till day 21 the activity increased very sharply to its third peak value. Thereof till day 30 the activity was on decline. Specific peroxidase activity also demonstrated three peak values during the culture period. The first peak was attained on day 6, second on day 12 and the third on day 21.

Table : 17. Growth, Polyphenols and progressive changes in the Activity of Peroxidase, IAA Oxidase, PAL and MDH during callus initiation from floral buds of N. tabacum L. var. Anand-2.
Medium : MS + 2.0 mg/l IAA + 2.0 mg/l NAA + 2.0 mg/l KN + 2% sucrose.
Incubation : 30 days at 26±2° in continuous light.
Inoculum : 500±60 mg fresh floral buds.

Day	Fresh weight mg/cult.	Dry weight mg/cult.	PEROXIDASE		IAA OXIDASE		MDH		PAL		PHENOLICS	
			units/ cult.	units/mg protein	units/ cult.	units/mg protein	units/ cult.	units/mg protein	units/ cult.	units/mg protein	mg/ cult.	mg%
0	500 (±60)	61.3 (±7)	-	-	-	-	31.19	9.66	-	-	1.84	3.01
3	678 (±52.8)	65.6 (±4)	0.33	0.05	-	-	58.91	10.33	-	-	1.18	1.81
6	866 (±49)	76.6 (±8.3)	4.65	0.69	-	-	25.28	3.80	-	-	1.04	1.37
9	1030.2 (±43)	97.1 (±10.6)	2.29	0.22	-	-	14.77	1.46	-	-	2.75	2.84
12	1259.0 (±68)	112.3 (±6.7)	2.66	0.51	-	-	80.69	15.68	2.62	0.51	3.48	3.10
15	1433.1 (±73)	134.3 (±12.1)	4.03	0.09	-	-	313.20	7.50	11.13	0.26	1.92	1.43
18	1440.0 (±86)	136.0 (±12)	3.57	0.31	3.19	0.28	70.95	6.30	2.98	0.26	1.10	0.81
21	1713.2 (±41)	189.2 (±14.4)	27.06	2.02	3.82	0.28	73.65	5.50	3.56	0.26	2.93	1.55
30	2104.4 (±68)	222.3 (±9.6)	14.72	1.08	1.34	0.09	49.79	3.67	3.61	0.26	4.15	1.87

Figures in parenthesis indicate standard error.
Data represented is average of 5 replicates.

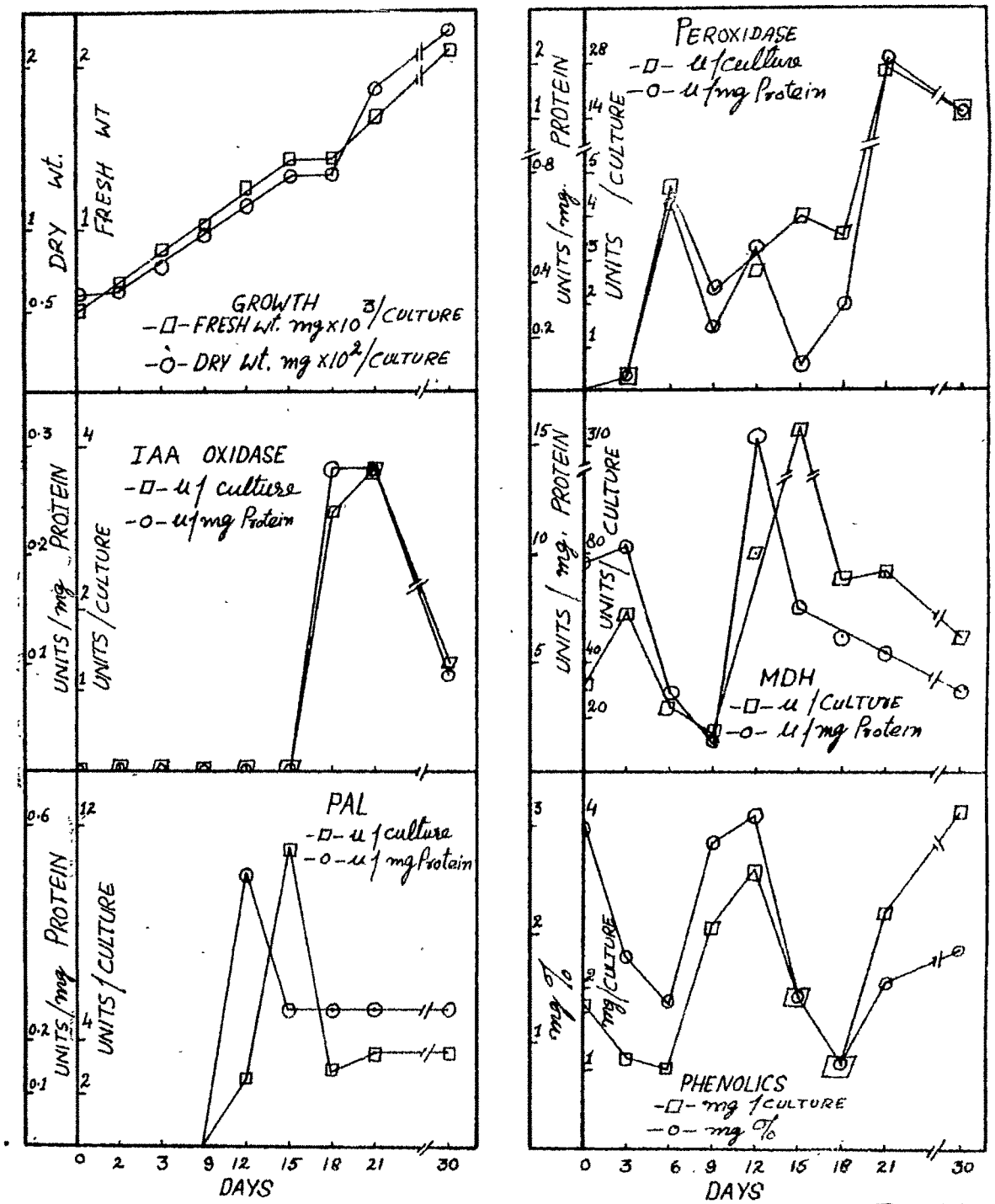


FIG. 79

Experimental Details as in Table 17.

Initiation of callus from floral buds on day 18 was followed by a sharp increase in peroxidase activity.

(c) IAA Oxidase :

Fig. 79 and Table 17 illustrate progressive changes in total and specific activity of IAA Oxidase in cultured floral buds of N. tabacum.

IAA Oxidase activity was not detectable till day 15. It was, however, detected from day 18 onwards. Total IAA Oxidase activity attained its peak value on day 21. Thereof till day 30 the activity declined sharply. On the other hand, specific IAA Oxidase attained its peak value on day 18. During subsequent three days i.e. till day 21, the enzyme activity remained stable, but thereafter it declined sharply till day 30.

It was noted that though the total IAA Oxidase activity was detected only after the initiation of callus on day 18, its specific activity showed correspondence with callus initiation (but not subsequent development).

(d) MDH :

Progressive changes of total and specific MDH activity in cultured floral buds of tobacco are illustrated in Fig. 79 and Table 17.

Total MDH activity increased rapidly during initial 3 days of culture, and first peak value was attained on day 3. Thereafter the activity was on decline till day 9. During day 9 and day 15 the activity increased tremandously to its second and major peak value. Thereof the activity declined sharply till day 18, remained more or less stable till day 21 and then continued to decline till day 30. This double peaked developmental pattern was exhibited by specific MDH activity also. The first peak value was reached on day 6 and the second on day 12. During day 12 and day 30 the activity was continuously on decline.

The MDH activity increased very sharply during the days immediately preceding callus initiation from floral buds (on day 18). With callus growth the activity decreased.

(e) PAL :

Fig. 79 and Table 17 illustrate progressive changes in total and specific PAL activity in the cultured floral buds of N. tabacum.

Till day 9 in culture no PAL activity could be detected. Thereafter, the enzyme activity was detectable and peak value attained on day 15. During day 15 and day 18 the activity declined sharply. From day 18 onwards till day 30 the activity was again on increase, but the increase in activity was rather marginal. Specific PAL activity reached its peak value on

day 12 of culture. Thereof it declined rapidly till day 15. During day 15 and day 30, specific PAL activity remained essentially stable. The PAL activity demonstrated sharp increase prior to callus initiation (on day 18). Thereafter it declined.

(f) Phenolics :

Fig. 79 and Table 17 illustrate progressive changes of phenolic content in the cultured flower buds of N. tabacum.

On culture basis the phenolic content in floral buds declined from 1.84 mg/culture on day 0 to 1.04 mg/culture on day 6. Thereafter it increased to 3.48 mg/culture by day 12. The phenolic content was again on decline during day 12 and day 18, the value being 1.10 mg/culture on day 18. During day 18 and day 30 the phenolics again started accumulating. On day 30 the total accumulation of phenolics was 4.15 mg/culture. On percentage basis the phenolic content in cultured floral buds declined from 3.01 mg% on day zero to 1.37 mg% on day 6. During day 6 and day 12 the phenolics accumulated and attained the value of 3.10 mg% on day 12. Thereof it declined to the value of 0.81 mg% by day 18. During day 18 and day 30 the phenolic content was on the increase, reaching the value of 1.87 mg% by day. 30. During six days preceding callus initiation i.e. day 12-18, the phenolic content of the cultured floral buds was on the decrease. However, the

phenolics started accumulating again with callus growth.

(g) Peroxidase Isoenzymes :

Fig. 79a illustrates progressive changes in the banding patterns of anodic and cathodic isoperoxidases in cultured floral buds of N. tabacum.

On day 0 of culture no anodic isoperoxidases were detected in peroxidase preparation of cultured floral buds of tobacco. Three slow migrating ones A_1, A_2 and A_3 were synthesized on day 3. Of these A_1 persisted till day 12, but A_2 and A_3 were seen again only on day 6. Three new anodic isoenzymes A_4, A_5 and A_6 were synthesized on day 6. A_4 persisted till day 12. A_5 remained suppressed for the rest of the culture period. A_6, however, was synthesized on day 9 also. On day 9 A_7, a slow migrating anodic isoenzyme, was synthesized. It never made appearance during the rest of the culture period. Another slow migrating one A_8 synthesized on the same day persisted till day 30. Four new anodic isoperoxidases A_9 - A_{12} were synthesized on day 12. A_9 and A_{11} were not synthesized on any other day during the culture period. A_{10} appeared on all days between days 12 and 30. A_{12} was suppressed on day 15 but reappeared on days 18, 21 and 30. A_{13} - A_{16}, four slow migrating anodic isoperoxidases were synthesized on day 15 of culture. A_{14} and A_{16} were not synthesized during the subsequent

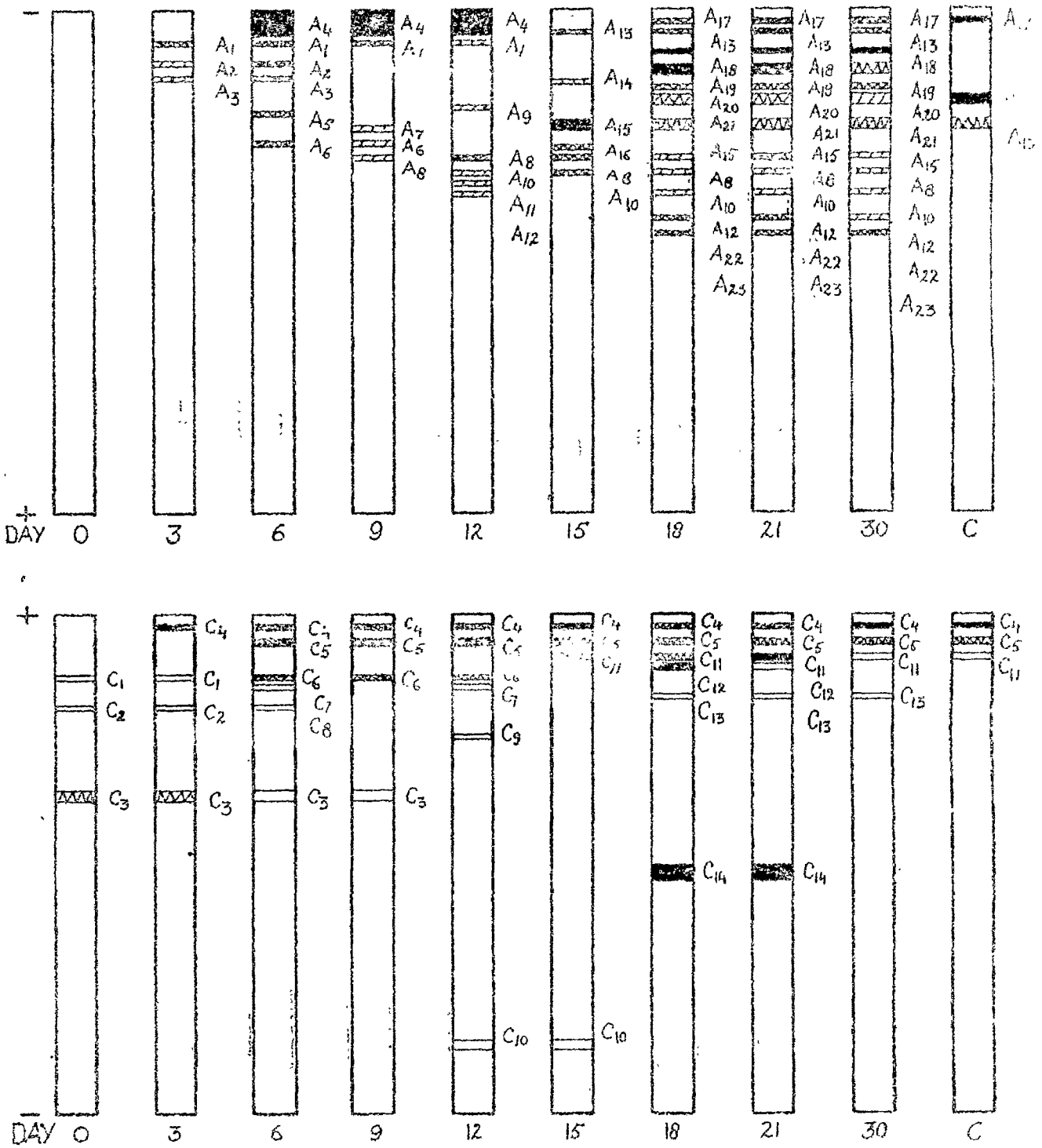


FIG. 79a.

Anodic and Cathodic Isoperoxidase Patterns. Experimental Details as in Table 17.

period of culture. A_{13} persisted right through till day 30. A_{15} also was synthesized on all days after day 15. Five slow migrating A_{17} - A_{21}, and two fast migrating A_{22} and A_{23}, anodic isoperoxidases were synthesized on day 18 and were also a part of the banding pattern on days 21 and 30. The slow migrating anodic isoperoxidases A_{17}, A_{21} and A_{15} were detected in detached callus masses formed from floral buds in culture. Of the three anodic isoperoxidases, A_{15}, A_{17} and A_{21}, detected in the callus masses of floral bud origin, only A_{15} was detected in cultured floral buds before callus became actually visible. The other two isoenzymes A_{17} and A_{21} were detected on day 18 i.e. only after callus was formed from the sepals of floral buds.

On the cathodic scale three slow migrating isoperoxidases C_1, C_2 and C_3 were present in floral buds on day 0. C_1 was synthesized on days 3-12, C_2 on days 13 and 6, and C_3 on days 3-9. On day 3 a slow migrating cathodic isoperoxidase C_4 was synthesized. It persisted right through the 30 day culture period. C_5 and C_6, two slow migrating cathodic isoperoxidases were synthesized on day 6. C_5 persisted right through the culture period. C_6 appeared again on day 12 only. C_7 a slow migrating isoperoxidase was synthesized on day 12. On the same day a fast migrating one C_8 too was synthesized, which reappeared on day 15. On day 15 a slow migrating cathodic

isoperoxidase C_9 was synthesized. It persisted for the remaining part of the culture period. C_{10} a slow migrating isoperoxidase was synthesized on days 18 and 21. C_{11} another slow migrating one was evident on days 18, 21 and 30. On days 18 and 21 a fast migrating cathodic isoperoxidase C_{12} was synthesized. In callus masses which originated from the cultured floral buds, three slow migrating cathodic isoperoxidases were present. These isoperoxidases were C_4, C_5 and C_9. Out of these C_4 was detectable in cultured floral buds on day 3, C_5 was detected on day 6 and C_9 on day 15. In other words, all the three isoperoxidases were synthesized in days prior to callus formation from the floral buds.

Expt. 24. Studies with floral bud callus of N. tabacum during its growth on standard medium.

The callus tissue initiated from floral buds of N. tabacum was subcultured regularly every 30 days to build up stock cultures. The medium used for optimum growth was MS basal medium supplemented with 2.0 mg/l each of IAA, NAA and KN, and 2% sucrose. 300±30 mg of fresh callus tissue was inoculated onto 40 ml of the above medium, which is referred to as the standard medium. The culture vessels were incubated at 26±2° in continuous light. Investigations carried out with floral bud callus during its growth on standard medium are documented below :

(a) Growth :

Growth measured as increments in fresh and dry weights of the floral bud callus is presented in Fig. 80 and Table 18.

The fresh weight increased linearly upto day 6 with 2.5 fold increase during this period. During days 6 and 9 fresh weight increased over 3 fold. Thereof till day 18 growth was rather slow, the callus mass having doubled during this 9 day period. Day 18 onwards saw another spurt in growth with a maximum yield of over 10 gm of fresh tissue. During the total 30 day period, growth increased 33.7 fold on fresh weight basis.

On dry weight basis growth of the callus tissues exhibited a period of slow growth during initial 6 days, followed by exponential growth till day 18, total foldwise increase being 17.36 during this 18 day period. Between days 18 and 30 dry weight increased only 2.33 fold, the total dry weight increase during 30 day culture period being 40.5 fold.

(ii) Peroxidase :

Peroxidase activity was assayed every three days in cell free extract of floral bud callus. The data is illustrated in Fig. 80 and Table 18.

The peroxidase activity remained rather low during the first 6 days in culture. Between days 6 and 18 the peroxidase activity increased sharply and linearly. However, the peak value for peroxidase activity was attained on day 21, after yet another sharp increase in the activity. By day 30 the activity had declined very rapidly.

Specific peroxidase activity exhibited marginal decline during initial 3 days of culture. The activity thereafter increased till day 9. The enzyme activity remained more or less steady. between days 9 and 18. Peak specific peroxidase activity was attained on day 21 in culture. By day 30 the activity had declined to the value as of day 0.

Table : 18. Growth, Polyphenols and progressive changes in the Activity of Peroxidase, IAA oxidase, MDH and PAL in floral bud callus of N. tabacum L. var. Anand-2 cultured on standard medium.
Medium : MS + 2.0 mg/l IAA + 2.0 mg/l NAA + 2.0 mg/l KN + 2% sucrose
Incubation : 30 days at 26±2° in continuous light
Inoculum : 300±30 mg fresh weight

Day	Fresh weight mg/cult.	Dry weight mg/cult.	PEROXIDASE		IAA OXIDASE		MDH		PAL		PHENOLICS	
			units/ cult.	units/mg protein	units/ cult.	units/mg protein	units/ cult.	units/mg protein	units/ cult.	units/mg protein	mg/ cult.	mg%
0	300 (±30)	12 (±3)	1.77	0.92	0.57	0.30	1.69	0.88	0.48	0.25	0.22	1.87
3	576 (±22.8)	42.5 (±6.1)	2.27	0.79	11.72	4.07	5.50	1.91	1.90	0.66	0.63	1.49
6	734.7 (±38.6)	44.6 (±4.0)	4.67	1.07	14.77	3.38	14.63	3.35	72.36	16.56	0.95	2.15
9	2411.2 (±48.1)	98.7 (±7.3)	15.55	1.18	31.23	2.37	39.80	3.02	127.05	9.64	1.52	1.55
12	3528.1 (±98.8)	133.3 (±8.6)	22.88	1.06	42.74	1.98	63.25	2.93	97.80	4.53	2.06	1.55
15	4194.8 (±120)	174.1 (±10)	35.52	1.14	50.79	1.63	53.59	1.72	46.42	1.49	2.92	1.68
18	4516.3 (±128)	208.4 (±9.5)	43.72	1.20	80.89	2.22	71.78	1.97	47.00	1.29	3.10	1.49
21	6268.4 (±214)	299.2 (±12.4)	138.13	2.32	99.43	1.67	144.08	2.42	109.55	1.84	6.43	2.15
30	10129.4 (±443.9)	486.1 (±16.3)	60.10	0.92	19.59	0.30	57.49	0.88	16.33	0.25	0.09	1.87

Figures in parenthesis indicate standard error.
Data represented is average of 5 replicates.

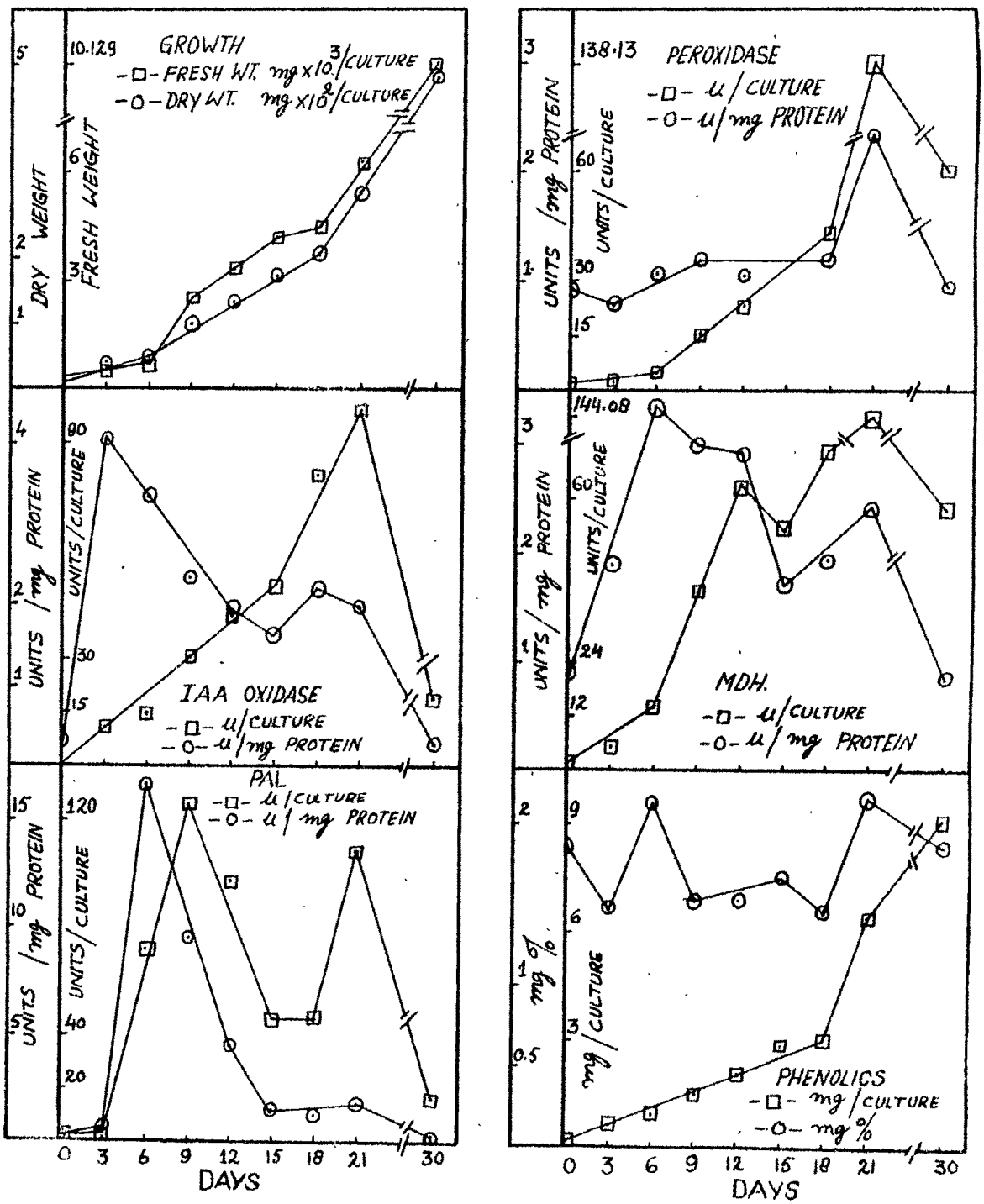


FIG. 80

Experimental Details as in Table 18.

(iii) IAA Oxidase :

Fig. 80 and Table 18 illustrate total and specific IAA Oxidase activity of floral bud callus cultured on the standard medium.

The total IAA Oxidase activity increased rapidly and linearly during the first 15 days of culture. The activity of IAA Oxidase shot up sharply between days 15 and 21, attaining its peak value on day 21. The activity of IAA Oxidase, however, thereof declined very rapidly till day. 30.

The specific activity of IAA Oxidase shot up during days 0 and 3, reaching its peak value. During the next twelve days i.e. till day 15 the activity, however, decreased unabated along a sharp gradient. On day 18 a slight increase in specific IAA Oxidase activity was registered over day 15 value. Thereafter the activity was on decline till day 30.

(iv) MDH :

Fig. 80 and Table 18 illustrate progressive changes in MDH activity in floral bud callus cultured on the standard medium.

The total Malate dehydrogenase activity in the floral bud callus increased rapidly during initial 12 days in culture, attaining its first peak value on that day. By day 15 the

activity decreased slightly, but from thereon it increased sharply to attain its second peak value on day 21. Beyond day 21 the activity declined till day 30.

There was linear increase in the specific activity of MDH between days 0 and 6, attaining its peak value on day 6. The activity declined between days 6 and 15, but increased again to attain its second peak value on day 21. The enzyme activity declined between days 21 and 30 to its value as of day 0.

(v) PAL :

The progressive changes in total and specific PAL activity during the growth of floral bud callus on the standard medium are illustrated in Fig. 80 and Table 18.

The PAL activity in floral bud callus tissue increased rather marginally during the initial 3 days of culture. Between days 3 and 9 the activity shot up immensely to attain its first peak value during the culture period. During the subsequent 6 days i.e. till day 15 the activity decayed very rapidly. During days 15 and 18 the activity remained essentially steady. Subsequent to rapid increase in the activity the second peak value was attained on day 21 of culture. The activity decayed very rapidly between days 21 and 30.

The specific activity of PAL increased marginally during the first 3 days in culture. Thereafter, it shot up to its peak value by day 6. From day 6 till day 15 the activity declined rapidly and linearly. The PAL activity remained more or less stable between day 15 and 21, but thereof declined till day 30 to its value as of day 0.

(vi) Phenolics

Fig. 80 and Table 18 illustrate progressive changes in the phenolic content of floral bud callus cultured on the standard medium.

The phenolic content in the floral bud callus increased steadily between days 0 and 18 from the value of 0.22 mg/culture to 3.10 mg/culture. During days 18 and 21 the phenolic content increased sharply to 6.43 mg/culture. By day 30 the total phenolic content was 9.09 mg/culture.

On percentage basis the phenolic content kept fluctuating during the entire culture period. During days 0 and 3 the value declined from 1.87 mg% to 1.49 mg%, from thereon it increased to 2.15 mg% by day 6. On day 9 the phenolic content declined again to 1.55 mg% and thereafter remained essentially steady till day 18, fluctuating between 1.49 and 1.68 mg%. By day 30 the phenolic content had, however, declined again to 1.87 mg%.

(vii) Peroxidase Isoenzyme :

Fig. 80a illustrates progressive changes in anodic and cathodic isoperoxidase banding patterns in floral bud callus tissues cultured on the standard medium.

Three anodic isoperoxidases A_1, A_2 and A_3 were present on day 0 of culture. The isoperoxidase A_1 remained suppressed during the initial 15 days of culture. It was synthesized on subsequent days of culture. The other isoenzyme A_2 was synthesized only on days 6, 21 and 30. The third original anodic isoperoxidase A_3 was synthesized on days 3, 6, 21 and 30. On all other days it remained suppressed. Five slow migrating anodic isoperoxidases A_4-A_7 and A_9 were also synthesized during the culture period. A_4 appeared between days 3-12; A_5 on day 3 only; A_6 on day 9 and 12; A_7 between days 9-18, and A_9 between days 15-21. A_8, a fast migrating anodic isoperoxidase, was synthesized on days 9 and 12 only.

On day 0 three slow migrating cathodic isoperoxidases C_1, C_2 and C_3 were present in the floral bud callus. Of these C_1 was synthesized on all days of culture; C_2 on days 6, 9, 18, 21 and 30. The third one C_3 remained suppressed till day 12. It was, however, synthesized on all days from day 15 onwards. Four slow migrating cathodic isoperoxidases C_4-C_6 and C_8 were synthesized during the culture period. C_4 was synthesized on days 3, 9 and 12; C_5 was evident on day 3 only; C_6 was

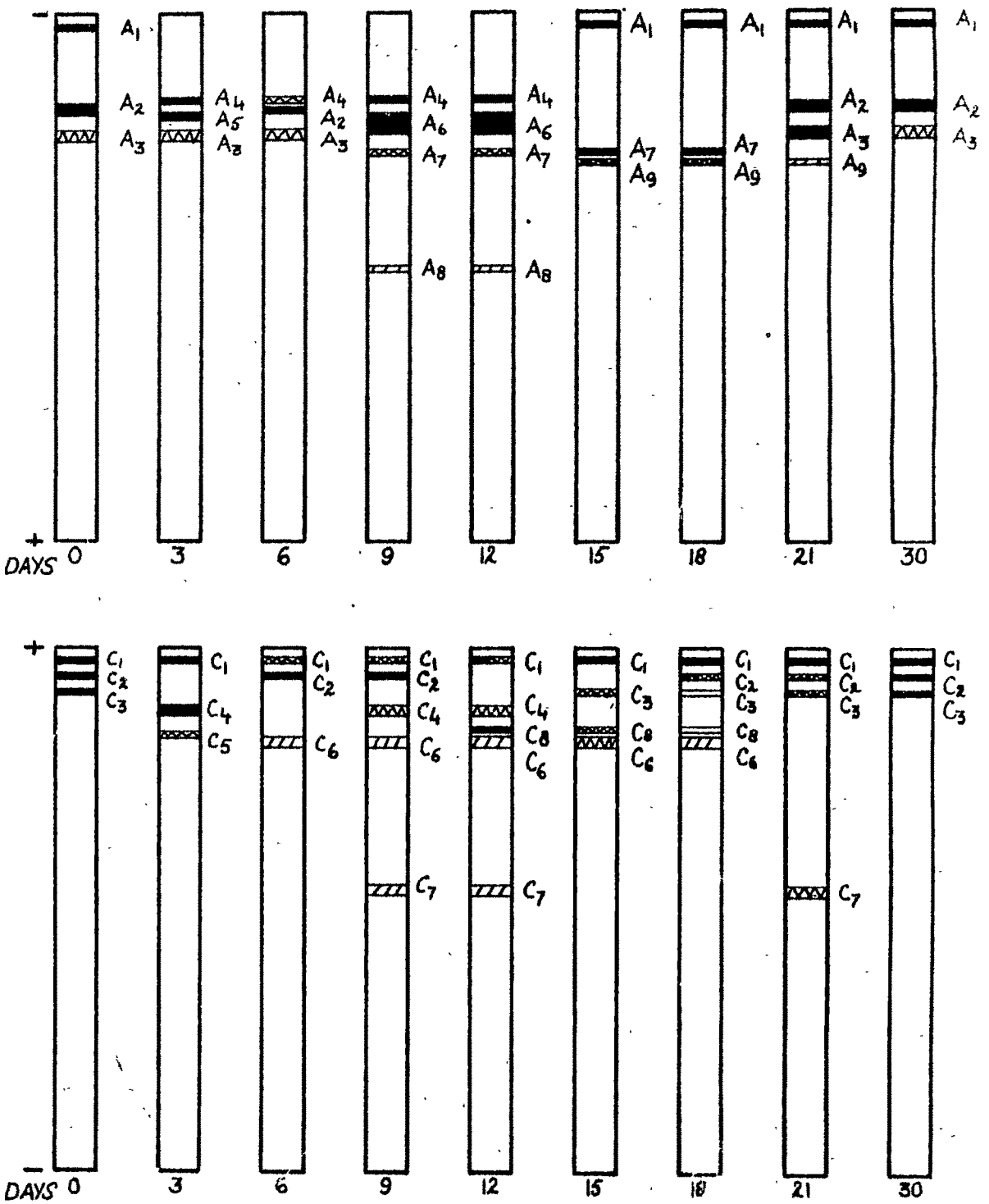


FIG. 80 a.

Anodic and Cathodic Isoperoxidase Patterns

Experimental Details as in Table 18.

synthesized between days 6-18; and C_8 on days 12, 15 and 18. A fast migrating cathodic isoperoxidase C_7 was synthesized on days 9, 12 and 21.

Summary :

Some correlations could be clearly made out between growth phases and the enzyme activity per unit protein of Peroxidase, IAA Oxidase, MDH and PAL. The peroxidase activity, with an initial slight decline in activity was on increase during the lag phase of growth. The activity remained more or less constant during the entire exponential phase. With the advent of post-exponential phase, the enzyme activity registered sharp increase attaining the highest peak during the entire growth period. At termination of the culture period, peroxidase activity returned to that observed at the beginning of lag phase.

The total IAA Oxidase activity per n unit protein was highest during the lag phase. With the onset of exponential phase and its progress, the enzyme activity was continuously on decline. On entering the post-exponential phase, total IAA Oxidase activity demonstrated slight increase in activity but by the termination of the culture period it had declined to the value observed at the beginning of lag phase.

The total MDH activity per unit protein was highest during the lag phase. During the exponential phase it was on decline. After the enzyme activity exhibited slight increase during late exponential period and early post-exponential phase it returned to its initial value.

The total PAL activity per unit protein attained its peak value during lag phase. Thereof, during the exponential and post- exponential phases the activity was continuously on decline.

The phenolic content in floral bud callus on percentage basis remained high during the lag and post- exponential phases. With the advent of exponential phase the phenolic content declined and remained more or less stable during rest of the period.

The original anodic isoperoxidase A_1 remained suppressed throughout the lag and early exponential phases. It was, however, synthesized during the late- exponential and post-exponential phases. The other two original anodic isoperoxidases A_2 and A_3 were strictly restricted to the lag and post exponential phases. Isoperoxidase A_7 was strictly restricted to the exponential growth phase. The staining intensity of the isoperoxidases reflect total peroxidase activity per unit protein. The inoculum (day 0) cathodic isoperoxidase C_1 was synthesized right through the growth cycle. C_2 was restricted

to lag and post- exponential phases. C_3 was synthesized during the late- exponential and post- exponential growth phases. C_6 was synthesized during the exponential phase and remained suppressed during the lag and post- exponential phases.

Expt. 25. Studies with haploid callus of N. tabacum during its growth on standard medium.

The haploid callus tissue initiated from stem explants of haploid plantlets of N. tabacum was subcultured regularly every 30 days to build up stock cultures. The medium used for optimum growth was MS basal medium supplemented with 2.0 mg/l each of IAA, NAA and KN, and 2% sucrose. 300±30 mg fresh callus tissue was inoculated onto 40 ml of the medium. The culture vessels were incubated at 26±2° in continuous light. Callus tissues were harvested every three days till day 21 and then on day 30 for analysis of growth, enzymes, isoperoxidase patterns and phenolic accumulation.

(i) Growth :

Growth of haploid callus tissues of N. tabacum measured as increase in fresh and dry weights, is illustrated in Fig. 81 and Table 19.

The fresh weight of haploid callus tissues increased from 0.3 g on day 0 to 1.44 g on day 9 i.e. 4.8 fold increase. Between days 9 and 30 the fresh weight increased from 1.44 g to 14.87 g i.e. an increase of 10.3 fold. During the entire culture period of 30 days fresh weight increased over 49 folds. The increase in dry weight followed an identical pattern. The growth was rather slow during the first 9 days but thereafter

it increased sharply and linearly. Dry weight increased over 47 folds during the 30 days culture period.

(ii) Peroxidase :

Progressive changes of total and specific peroxidase activity in haploid callus during its growth on the standard medium are illustrated in Fig. 81 and Table 19.

Total peroxidase activity in haploid callus tissues grown on the standard medium was on increase from the very outset. The peak value was reached on day 21 of culture. By day 30 the activity declined. On the other hand, specific peroxidase activity declined slightly by day 3 in culture. Between days 3 and 9 the specific peroxidase activity increased sharply to reach peak value. Between days 9 and 21 the activity was on decline along a linear gradient, but the rate of decay of enzyme activity was slow. Between days 21 and 30 the enzyme activity decayed very sharply.

(iii) IAA Oxidase :

Progressive changes of IAA Oxidase activity during the growth of haploid tobacco callus tissue on standard medium are presented in Fig. 81 and Table 19.

The total activity of IAA Oxidase increased gradually and linearly from day 0 till day 9. From day 9 till day 18

Table : 19. Growth, Polyphenols and progressive changes in the Activity of Peroxidase, IAA oxidase, MDH and PAL in Haploid callus of N. tabacum L. var. Anand-2 cultured on standard medium.
Medium : MS + 2.0 mg/l IAA + 2.0 mg/l NAA + 2.0 mg/l KN + 2% sucrose
Incubation : 30 days at 26±2° in continuous light
Inoculum : 300±30 mg fresh weight

Day	Fresh weight mg/cult.	Dry weight mg/cult.	PEROXIDASE		IAA OXIDASE		MDH		PAL		PHENOLICS	
			units/ cult.	units/mg protein	units/ cult.	units/mg protein	units/ cult.	units/mg protein	units/ cult.	units/mg protein	mg/ cult.	mg%
0	300 (±30)	12 (±3)	0.98	0.86	0.48	0.42	1.05	0.92	0.28	0.25	0.13	1.12
3	604.4 (±44)	26.9 (±2.8)	2.38	0.79	10.96	3.63	3.11	1.03	2.65	0.88	0.98	3.67
6	1041.4 (±86.7)	48.6 (±4.4)	6.09	1.50	14.53	3.58	10.79	2.66	62.32	15.35	1.38	2.84
9	1443.7 (±110.3)	68.0 (±4.2)	16.72	1.84	32.08	3.53	30.17	3.32	88.35	9.72	1.46	2.15
12	3542.9 (±163.0)	132.8 (±8.0)	19.06	1.74	50.99	3.05	46.81	2.80	39.29	2.35	2.39	1.81
15	6291.2 (±203.0)	214.8 (±12.6)	53.88	1.66	90.56	2.79	98.02	3.02	13.63	0.42	3.07	1.43
18	7869.9 (±398.5)	284.5 (±18.2)	61.11	1.50	141.67	2.55	384.47	6.92	23.33	0.42	4.06	1.43
21	9378.4 (±538.0)	426.9 (±30.1)	72.56	1.46	35.78	0.72	268.87	5.41	17.89	0.36	6.10	1.43
30	14879.2 (±865.9)	564.3 (±42.0)	48.87	0.86	23.86	0.42	52.28	0.92	14.20	0.25	6.32	1.12

Figures in parenthesis indicate standard error.
Data represented is average of 5 replicates.

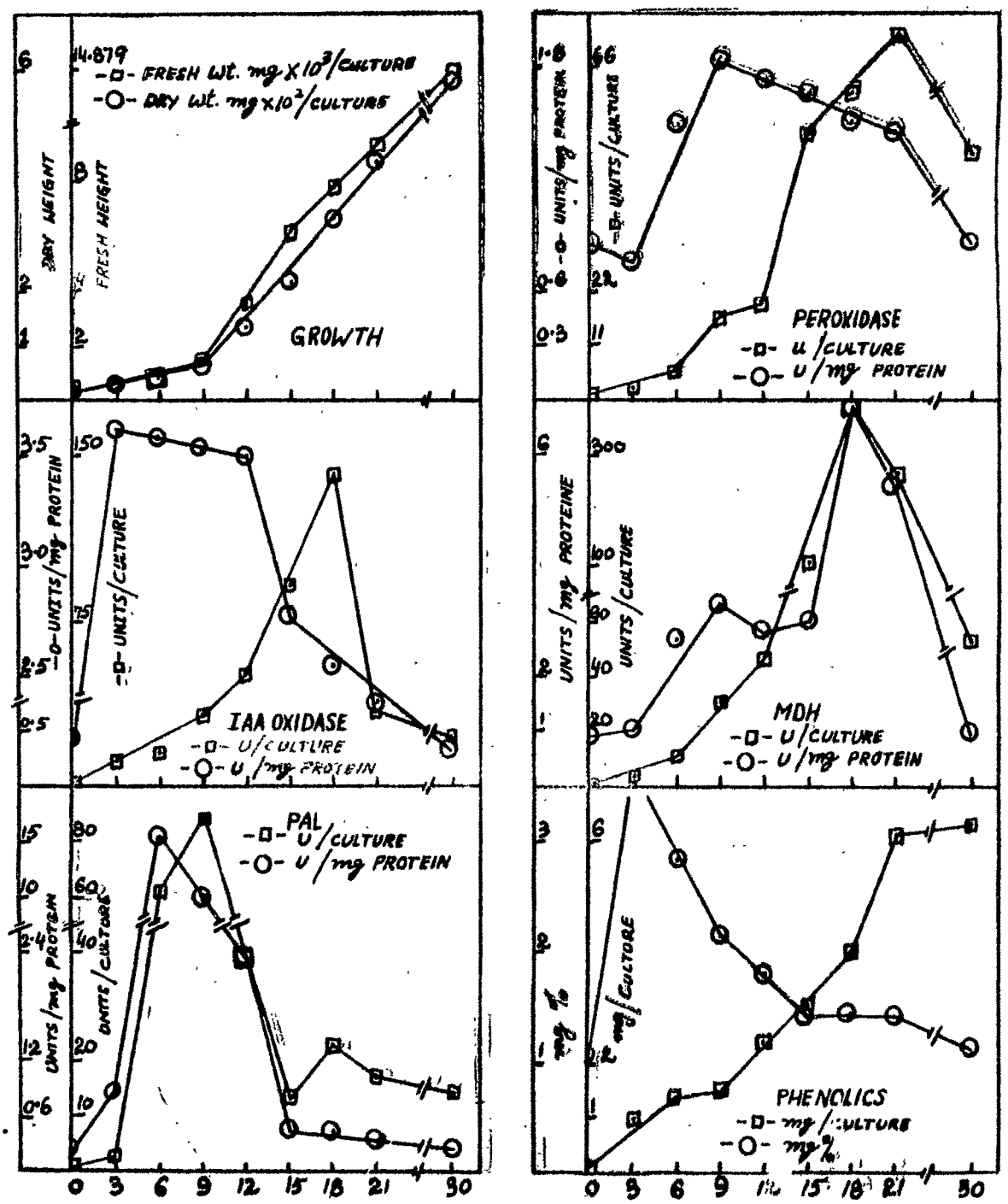


FIG. 81

Experimental Details as in Table 19.

the increase in the activity was phenomenal and reached the peak value on day 18. By day 21 the IAA Oxidase activity dropped very sharply and continued to decline till day 30. On the other hand, specific activity of IAA Oxidase attained its peak value on day 3. Thereafter specific IAA Oxidase activity declined till day 30. Between days 3 and 12 the activity declined along a slow but linear gradient, followed by sharp decline till day 15. Between days 15 and 30 the specific activity continued to decline linearly.

(iv) MDH :

The progressive changes of total and specific activities of MDH in haploid tobacco callus tissue during its growth on standard medium are illustrated in Fig. 81 and Table 19.

The total MDH activity in haploid tobacco callus grew very rapidly and attained its peak value on day 18, followed by sharp decline till day 30. The activity increased more than 350 folds between days 0 and 18. The specific MDH activity demonstrated double peak pattern of development. The first peak value was reached on day 9 and the second on day 18. Between days 18 and 30 the activity declined very sharply.

(v) PAL :

The progressive changes of PAL activity in haploid tobacco callus during its growth on standard medium are

illustrated in Fig. 81 and Table 19.

The total PAL activity showed a double peak pattern. The total activity increased slightly by day 3. Thereafter, a sharp increase ensued till day 9 in the PAL activity. Between days 9 and 15 the total PAL activity declined sharply and linearly. By day 18, however, it increased to a second peak only to decline again till day 30. The specific activity of PAL demonstrated only one peak value, which was reached on day 6. Thereafter the activity declined sharply till day 15. Between days 15 and 30 the activity continued to decline but the changes were slight.

(vi) Phenolics :

The progressive changes of phenolic accumulation in haploid tobacco callus cultures during its growth on standard medium are illustrated in Fig. 81 and Table 19.

On culture basis the accumulation of phenolics was on increase from day 0 till day 30. The peak value of 6.32 mg/culture was reached on day 30. On the other hand, phenolic accumulation on percentage basis reached its peak value of 3.67 mg% on day 3. Thereafter it continued to decline till day 30.

(vii) Peroxidase Isoenzymes :

The progressive changes of the banding patterns of anodic

and cathodic isoperoxidases during growth of haploid tobacco callus on standard medium are illustrated in Fig. 81a.

To start with three anodic isoperoxidases A_1, A_2 and A_3 were present in the haploid callus tissue. Till day 15 the anodic isoperoxidase A_1 remained suppressed. A_2 and A_3 were seen on day 3. A_3 reappeared from day 15 onwards, whereas A_2 reappeared on day 30. During the course of culture five slow migrating anodic isoperoxidases were synthesized. Of these A_4 was synthesized on days 3 and 6, A_6 on days 6 and 12, A_7 on days 6, 15, 18 and 21, A_8 on days 9 and 12, and A_9 on days 9 and 12. Only one fast migrating anodic isoperoxidase A_5 was synthesized on days 3, 9 and 12.

In haploid tobacco tissues the cathodic isoperoxidases too were three, C_1, C_2 and C_3. C_1 persisted right through the culture period. C_2 was synthesized only on days 12, 21 and 30. C_3 appeared on days 3, 18, 21 and 30. Nine cathodic isoperoxidases, all slow migrating, were synthesized at varying intervals during the culture period. C_4 appeared on days 3, 6 and 9, C_5 on day 6, C_6 on days 9 and 18, C_7 on day 9, C_8 on day 12, C_9 on days 12 and 21, C_{10} on days 15 and 18, C_{11} on day 15, and C_{12} on days 18 and 21.

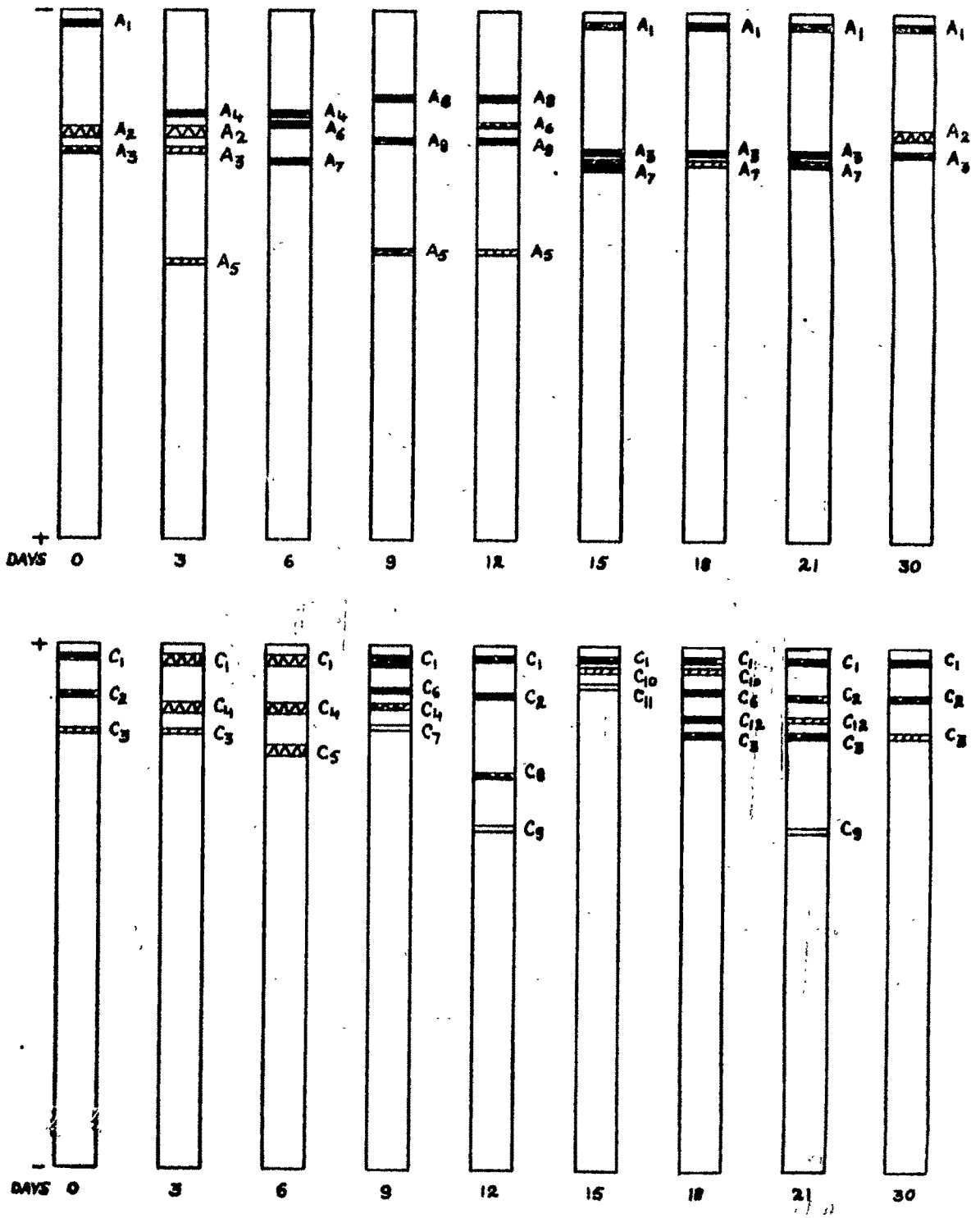


FIG. 81 a.

Anodic and Cathodic Isoperoxidase Patterns.
Experimental Details as in Table 19.

Summary :

Unlike the growth of diploid tobacco callus tissues, no lag phase was noticed in the growth cycle of haploid callus tissues. The exponential phase lasted initial 9 days and was followed by post- exponential phase till day 30.

The total peroxidase activity per unit protein declined slightly during early exponential phase, for the rest of exponential growth phase the activity was on increase. With the advent of post- exponential growth phase the enzyme activity started to decline and continued to decay till the end of culture period. In haploid callus tissues the period of highest peroxidase activity was the exponential growth phase whereas it was the post- exponential phase in case of diploid callus tissues. In haploid callus tissues the enzyme activity during exponential phase was on increase, whereas in diploid callus tissues it remained more or less stable.

The total IAA Oxidase activity per unit protein increased sharply during the early exponential phase, but with its progress the enzyme activity was on decline along a slow linear gradient. The activity continued to decay during the post-exponential growth phase, but at a much faster rate than observed during the earlier phase. The IAA Oxidase activity in diploid and haploid callus tissues followed essentially similar developmental patterns.

The total MDH activity per unit protein in haploid tobacco callus tissues was on increase during the entire exponential phase, it recorded the highest activity in post-exponential period of growth. Here again as in the case of peroxidase, the enzyme pattern was unlike that observed in diploid callus tissues. In diploid callus maximum MDH activity was registered during the lag phase and the enzyme activity was on decline during the exponential phase of growth.

The total PAL activity per unit protein in haploid callus tissues registered its highest value for the entire growth cycle during early exponential phase. During the late-exponential and post- exponential periods the enzyme activity was on decline. Decline in the activity was very sharp during the early part of post-exponential phase. In contrast, the PAL activity in diploid callus tissues registered peak value during the lag period and thereof was continuously on decline during the entire exponential and post-exponential periods.

During the early phase of exponential period, maximum phenolic accumulation (on percent basis) was achieved. With the progress of exponential phase and in the post-exponential period the phenolic content decreased. On the other hand, in diploid callus tissues maximum phenolic accumulation was achieved during the post-exponential phase. Further the phenolic content remained more or less constant during the

exponential growth phase in diploid callus tissues.

As in diploid callus tissues the initial anodic isoperoxidase A_1 was restricted to the post-exponential phase of growth in haploid callus also. This particular isoperoxidase exhibited identical electrophoretic mobility in both the tissues. Another haploid anodic isoperoxidase A_2 (which exhibited similar electrophoretic mobility as A_3 in diploid callus), was also essentially restricted to the post-exponential phase of growth. As in the case of diploid callus the original cathodic isoperoxidase C_1 was present throughout the growth cycle in haploid callus tissues too. The original cathodic isoperoxidase C_3 was restricted essentially to the fag end of the post-exponential phase of growth. No particular isoperoxidase appeared with consistency during any specific phase of growth.

Section E : Physiological Studies with Peroxidase, IAA Oxidase, MDH, PAL, Peroxidase Isoenzymes and Phenolics in Diploid and Haploid callus tissues of *N. tabacum* cultured on root differentiating medium.

From the experiments carried out earlier and described in Sections C-I and C-II of Chapter III (Results), it became obvious that diploid and haploid callus cultures of N. tabacum retained their morphogenetic potential in vitro. The organogenetic responses of these callus cultures rested largely on the exogenous supply of phytohormones, singly or in combinations, and the subtle ratio between them.

To examine the physiological changes associated with root differentiation in diploid and haploid callus tissues of N. tabacum the following parameters were studied : (i) Growth, (ii) Peroxidase, (iii) IAA Oxidase, (iv) MDH, (v) PAL, (vi) Peroxidase Isoenzyme patterns and (vii) Phenolic accumulation.

The root differentiating medium used for study was : MS (basal) + 2.0 mg/l IAA + 3% sucrose. Roots were induced on this medium within a maximum of 15 days from diploid callus and within 9 days from haploid callus tissues with over 75 per cent frequency. The experiments were terminated as soon as morphogenic responses were manifested or else were carried on till day 15 of culture. During this period the callus tissues were harvested every third day, commencing with day 0, till day 15 and analysed for the parameters enlisted above.

As mentioned earlier on in Chapter I (Introduction) level of sucrose in the medium is reported to influence morphogenetic

response of tissue cultures. It also became clear in the present study (Chapter III, Results, Sections C-I and C-II) that the level of sucrose not only altered the degree of response but also influenced the time taken for its expression.

Earlier studies mentioned in Chapter I (Introduction) indicated that hydroxylated aromatic compounds like phenolic acids influenced organogenesis in cultured tissues. It was shown that their effectiveness could be correlated with their enhancement of peroxidase mediated IAA Oxidation. It was proposed that certain phenols stimulated IAA inactivation, thus lowering the physiological level of IAA in the plant tissue and creating a situation more favourable for organogenesis. Certain phenols have also been acredited with the function of being auxin protectors, thereby preventing its destruction. In the present investigation, experiments were conducted to examine the effect/s of some phenolic acids on root differentiation from both diploid and haploid callus. Associated physiological changes were also studied.

On account of varied physiological roles attributed to Mn^{++}, which are already elaborated in the Introduction (Chapter I), effects of different doses of Mn^{++} on root differentiation and associated physiological parameters were also looked into.

For the sake of convenience, results in this section are presented under following heads :

(i) Studies with floral bud callus cultured on root inducing medium,

(ii) Studies with floral bud callus cultured on root inducing medium supplemented with low and high levels of sucrose,

(iii) Studies with floral bud callus cultured on root inducing medium supplemented with various phenolic acids,

(iv) Studies with floral bud callus cultured on root inducing medium containing $\frac{1}{2}$ x and 2 x Mn^{++} ion concentration,

(v) Studies with haploid callus tissues cultured on root inducing medium,

(vi) Studies with haploid callus tissues cultured on root inducing medium supplemented with low and high levels of sucrose,

(vii) Studies with haploid callus tissues cultured on root inducing medium supplemented with various phenolic acids,

(viii) Studies with haploid callus tissues cultured on root inducing medium containing $\frac{1}{2}$ x and 2 x Mn^{++} ion concentrations.

Expt. 26. Studies with floral bud callus cultured on root inducing medium.

From the stock cultures healthy looking, lush green callus masses weighing 300±30 mg were selected and cultured on 20 ml MS basal medium supplemented with 2.0 mg/l IAA and 3.0% sucrose. The culture vessels were incubated at 26±2° in continuous light. The roots differentiated from the callus tissues between days 13 and 15. Every three days five replicates were harvested and analysed for growth, enzymes, isoperoxidase patterns and phenolic content. The results are described below.

(a) Growth :

Growth was measured as increase in fresh and dry weights and is illustrated in Fig. 82 and Table 20.

Growth on both fresh and dry weight basis exhibited typical double sigmoid curve. Upto day 3 in culture a lag phase was observed followed by 3 days of rapid growth. During this period fresh weight increased approximately 1.5 fold and dry weight by 2.5 fold. Another slow period of growth was observed between days 6 and 9. Thereafter growth increased linearly till day 15. During the entire culture period fresh weight increased 1.85 fold and dry weight by 5.11 fold.

From the cultured callus masses roots were differentiated

anytime between days 13 and 15. The frequency of response was around 75%. Per callus mass 4-6 roots were differentiated.

(b) Peroxidase :

The development of peroxidase activity is represented in Fig. 82 and Table 20.

During first 3 days in culture the peroxidase activity decreased to nearly half its value as of day 0. From day 3 onwards till day 9 the enzyme activity increased attaining its first peak value. On day 12 the enzyme activity dropped again but by day 15 increased to register its second peak value.

Specific peroxidase activity also declined during first 3 days of culture. Between days 3 and 9 the enzyme activity increased to reach its peak value. The specific activity declined by day 12 and thereof started to increase again till day 15.

Both total and specific peroxidase activities were on the increase just before the differentiation of roots on day 15.

(c) IAA Oxidase :

The progressive changes of IAA Oxidase activity are illustrated in Fig. 82 and Table 20.

Total IAA Oxidase activity increased gradually between days 0 and 6. Between days 6 and 12 the enzyme activity did

Table : 20. Growth, Polyphenols and progressive changes in the Activity of Peroxidase, IAA oxidase, MDH and PAL during root differentiation from Diploid callus tissues of N. tabacum L. var. Anand-2.

Medium : MS + 2.0 mg/l IAA + 3% sucrose

Incubation : 15 days at 26±2° in continuous light

Inoculum : 300±30 mg fresh weight

Day	Fresh weight mg/cult.	Dry weight mg/cult.	PEROXIDASE		IAA OXIDASE		MDH		PAL		PHENOLICS	
			units/ cult.	units/mg protein	units/ cult.	units/mg protein	units/ cult.	units/mg protein	units/ cult.	units/mg protein	mg/ cult.	mg%
0	300 (±30)	12 (±3)	1.77	0.92	0.57	0.30	1.69	0.88	0.48	0.25	0.22	1.87
3	309.6 (±22.0)	15.2 (±2.6)	1.05	0.71	0.92	0.62	0.98	0.66	0.55	0.37	0.42	2.80
6	438.5 (±41.2)	38.4 (±6.2)	1.81	0.84	1.65	0.77	2.11	0.99	0.68	0.32	1.36	3.55
9	460.9 (±36.0)	40.6 (±5.0)	2.77	1.16	1.74	0.73	0.76	0.32	1.04	0.44	1.64	4.05
12	499.8 (±32.4)	50.3 (±4.3)	1.58	0.79	1.56	0.78	0.53	0.26	2.98	1.49	1.91	3.80
15	557.9 (±51.2)	61.4 (±7.7)	3.53	0.83	2.51	0.62	10.73	2.66	1.93	0.48	1.71	2.80

Figures in parenthesis indicate standard error.

Data represented is average of 5 replicates.

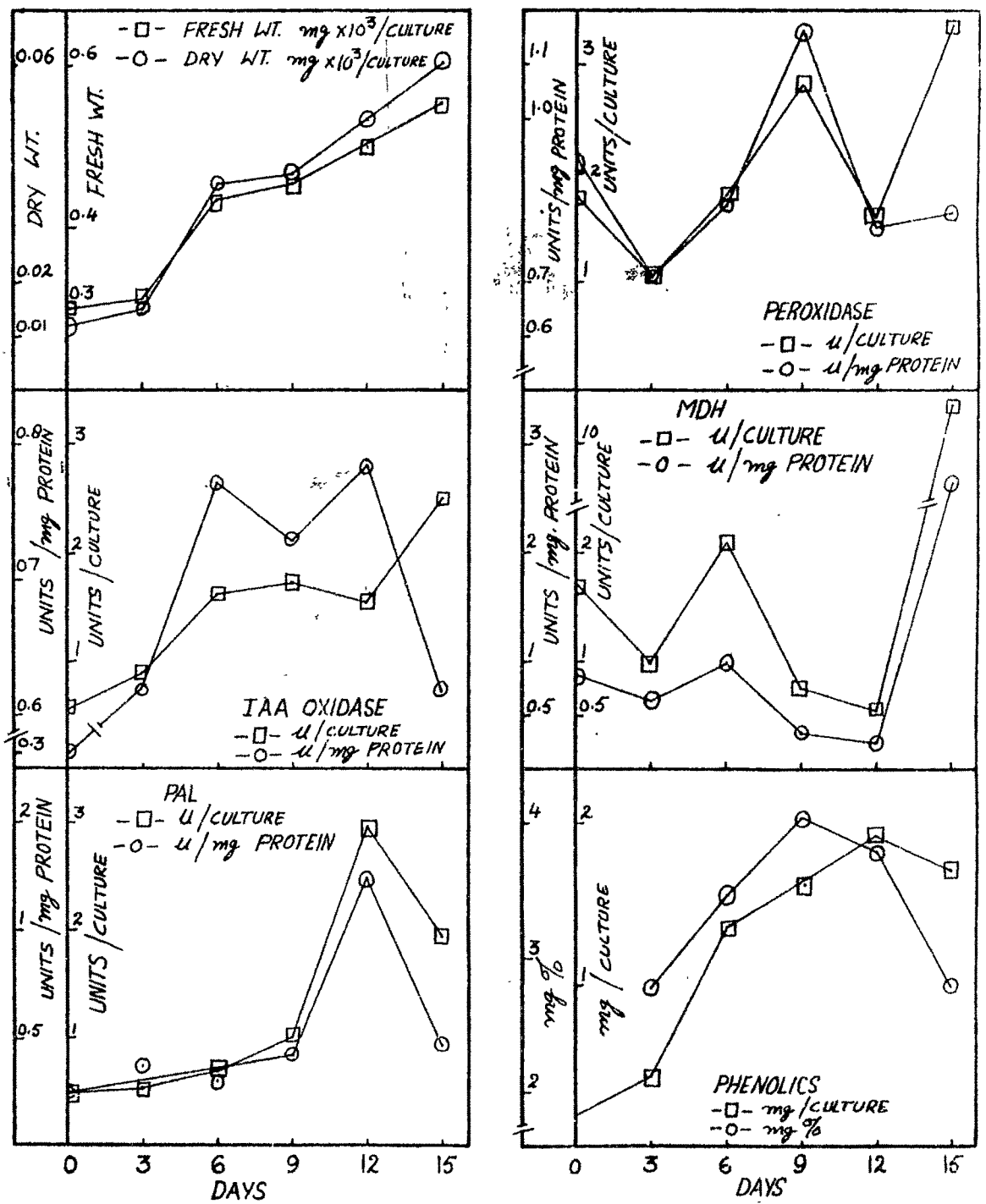


Fig. 82.

Experimental Details as in Table 20.

not change significantly. The enzyme activity, however, shot up between days 12 and 15.

Specific IAA Oxidase activity increased nearly 3 fold between days 0 and 6. The activity declined slightly by day 9, but increased again to achieve its peak value on day 12, before dropping again by day 15.

Just before the differentiation of roots on day 15 though the total IAA Oxidase activity was on the increase, the specific activity demonstrated a sharp decline.

(d) MDH :

The progressive changes in enzyme activity of MDH are illustrated in Fig. 82 and Table 20.

The total activity of MDH declined between days 0 and 3 from 1.69 units to 0.98 units. The activity, however, increased again to 2.11 units by day 6. Between days 6 and 12 the enzyme activity dropped to an all time low of 0.53 units, but by day 15 had jumped up nearly 20 fold.

The specific activity of MDH followed essentially the same pattern of development as total activity. However, changes in specific activity from time to time were not as pronounced as the total activity.

The appearance of roots from callus tissues on day 15 was preceded by a sharp increase in activity of MDH.

(e) PAL :

The PAL activity in cell free extract of flower bud callus during differentiation of roots is illustrated in Fig. 82 and Table 20.

There was no significant increase in PAL activity during the days 0 and 3. During days 3 and 9 the activity increased linearly by 2 fold. The activity on day 12 shot up to reach its peak value before dropping to half its value on day 15.

The specific activity of PAL fluctuated slightly during days 0 and 9. Between days 9 and 12 the activity shot up to a peak value which was nearly 4 times more than day 9 activity. The activity, however, declined on day 15.

The total and specific activities of PAL reached the peak values prior to differentiation of roots.

(f) Phenolics :

Variation in phenolic content of floral bud callus cultures during root differentiation are illustrated in Fig. 82 and Table 20.

The phenolic content of floral bud callus during root initiation increased between days 0 and 12 from 0.22 mg/culture to 1.91 mg/culture. On day 15 the value decreased marginally to 1.71 mg/culture. On percentage basis the accumulation of phenolics increased between days 0 and 9 from 1.87 mg% to

4.05 mg%. The phenolic content, however, declined between days 9 and 15 to 2.8 mg%.

Though the phenolic content increased between days 0 and 9, it started to decline just before differentiation of roots.

(g) Peroxidase Isoenzymes :

The progressive changes of anodic and cathodic isoperoxidases during root differentiation from floral bud callus of tobacco are illustrated in Fig. 82a.

Of the three initial anodic isoperoxidases only A_1 and A_2 persisted throughout the culture period. The third isoperoxidase A_3 did not make its appearance from day 3 onwards. Two anodic isoperoxidases A_4 and A_5 appeared on day 3, of these A_5 persisted till the end of culture period; whereas, A_4 remained suppressed on days 6 and 9, but was synthesized again on days 12 and 15. Day 6 heralded the synthesis of isoperoxidases A_6 and A_7. Isoperoxidase A_6 was present on day 9 also but A_7 was never seen after day 6. On day 12 the isoenzyme PR was synthesized before roots were differentiated or rather before the visual manifestation of root differentiation. The isoperoxidase PR had relative mobility (Rm) of 0.3. Disc gel electrophoretic analysis of detached regenerated root isoperoxidases revealed this characteristic isoperoxidase PR which,

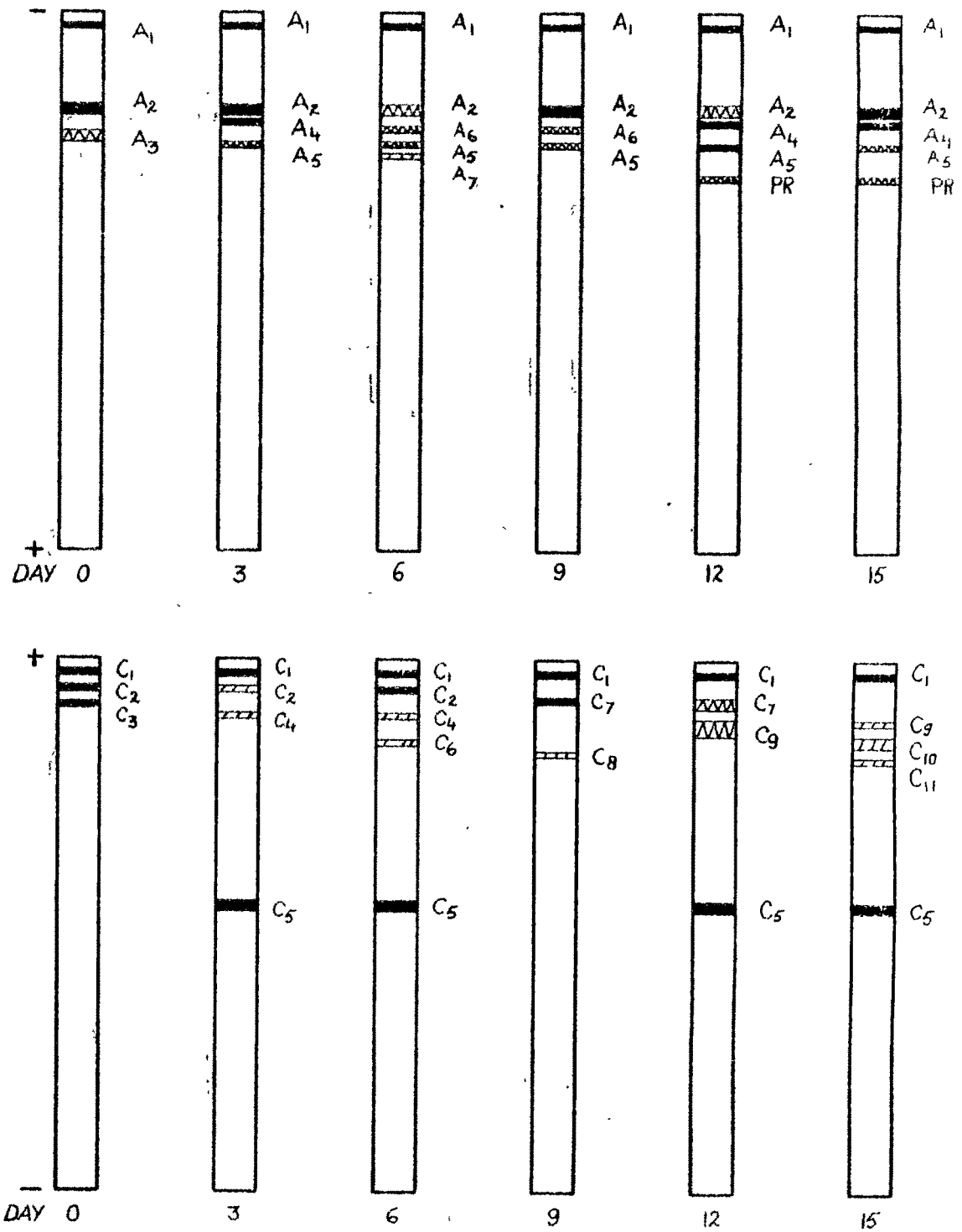


Fig. 82 a.

Anodic and Cathodic Isoperoxidase Patterns.

Experimental Details as in Table 20

however, was absent in the callus tissues.

To start with on day 0 of culture, three cathodic isoperoxidases C_1, C_2 and C_3 were present in floral bud callus. Only isoperoxidase C_1 persisted throughout the culture period. Isoperoxidase C_2 remained till day 6 and isoperoxidase C_3 was repressed from day 0 onwards. On day 3 one slow migrating isoperoxidase C_4 and one fast migrating C_5 were synthesized. The isoperoxidase C_4 was repressed from day 9 onwards; whereas, isoperoxidase C_5 appeared on all days except day 9. During the culture period other slow migrating cathodic isoperoxidase made appearance, C_6 on day 6, C_7 on days 9 and 12, C_8 on day 9, C_9 on days 12 and 15, and C_{10} and C_{11} on day 15. No correlation was found between the cathodic isoperoxidase banding pattern and root differentiation.

Expt. 27. Studies with floral bud callus cultured on root inducing medium supplemented with low and high levels of sucrose.

Healthy callus masses of N. tabacum weighing 300±30 mg by fresh weight were cultured on 20 ml of rooting medium (MS + 2.0 mg/l IAA) in which the levels of sucrose used were low (1 per cent) and high (6 per cent), and are referred to in the text as Medium A and Medium B respectively. The culture vessels were incubated at 26±2° in continuous light for a period of 15 days. Every three days 5 replicates were harvested and analysed for growth, enzymes, isoperoxidase banding patterns and phenolic content.

(a) Growth :

Growth measured as increase in fresh and dry weight is illustrated in Figs. 83, 84 and Tables 21, 22.

The growth of floral bud callus on Medium A (i.e. 1% sucrose containing medium) was better than on Medium B (i.e. 6% sucrose medium). On the former fresh weight increased 4.4 fold and dry weight by 7.75 folds (Fig. 83, Table 21). On the latter fresh and dry weight increases were 1.97 and 4.4 fold respectively (Fig. 84, Table 22). Moreover, the differentiation of roots was completely absent on Medium A. On Medium B roots differentiated on day 12 of culture but never more than two

per callus mass, with about 25 per cent frequency.

(b) Peroxidase :

The development of peroxidase activity is illustrated in Figs. 83, 84 and Tables 21, 22.

On Medium A total peroxidase activity after fluctuating slightly during the first 6 days started ascending and attained its peak value on day 12; but by day 15 it again declined. On the contrary, on Medium B the enzyme activity was on decrease during the first 9 days. From thereon it increased to reach its peak value on day 15. On any given day total peroxidase activity was appreciably higher on Medium A than on Medium B.

The specific activity of Peroxidase attained its peak value on day 3 on both the media. On Medium A second peak was attained on day 9, whereas it was attained on day 15 on Medium B. The peak value of peroxidase on day 3 on Medium B is more than double that on Medium A.

On medium A (i.e. the root differentiating medium supplemented with 1% sucrose) the total and specific peroxidase activity was on the decline during the last phase of the culture. In contrast, it was on the increase from day 9 onwards on Medium B (i.e. 6% sucrose containing medium) whereon root differentiation occurred on day 12.

Table : 21. Growth, Polyphenols and progressive changes in the Activity of Peroxidase, IAA Oxidase, MDH and PAL in Diploid callus cultures of N. tabacum L. var. Anand-2 cultured on root differentiation medium containing sub-optimum level of sucrose.

Medium : MS + 2.0 mg/l IAA + 1% sucrose

Incubation : 15 days at 26±2° in continuous light

Inoculum : 300±30 mg fresh weight

Day	Fresh weight mg/cult.	Dry weight mg/cult.	PEROXIDASE		IAA OXIDASE		MDH		PAL		PHENOLICS	
			Units/ cult.	units/mg protein	units/ cult.	units/mg protein	units/ cult.	units/mg protein	units/ cult.	units/mg protein	mg/ cult.	mg%
0	300 (±30)	12 (±3)	1.77	0.92	0.57	0.30	1.69	0.88	0.48	0.25	0.22	1.87
3	696.4 (±48)	39.0 (±6)	2.64	2.32	4.22	3.70	7.21	6.33	1.54	1.35	0.64	1.65
6	765.3 (±28.2)	41.5 (±5.1)	1.76	0.61	5.35	1.87	19.91	6.98	23.80	8.34	0.52	1.27
9	816.2 (±33.6)	57.3 (±3.8)	3.50	1.47	0.91	0.39	18.06	7.62	15.41	6.49	0.73	1.27
12	1032.6 (±52.7)	72.4 (±7.0)	11.08	0.93	8.01	1.32	18.19	3.01	22.21	3.68	1.10	1.52
15	1325.4 (±62.9)	93.0 (±4.7)	5.03	0.55	20.72	2.26	18.31	1.98	8.82	0.96	0.61	0.65

Figures in parenthesis indicate standard error.

Data represented is average of 5 replicates.

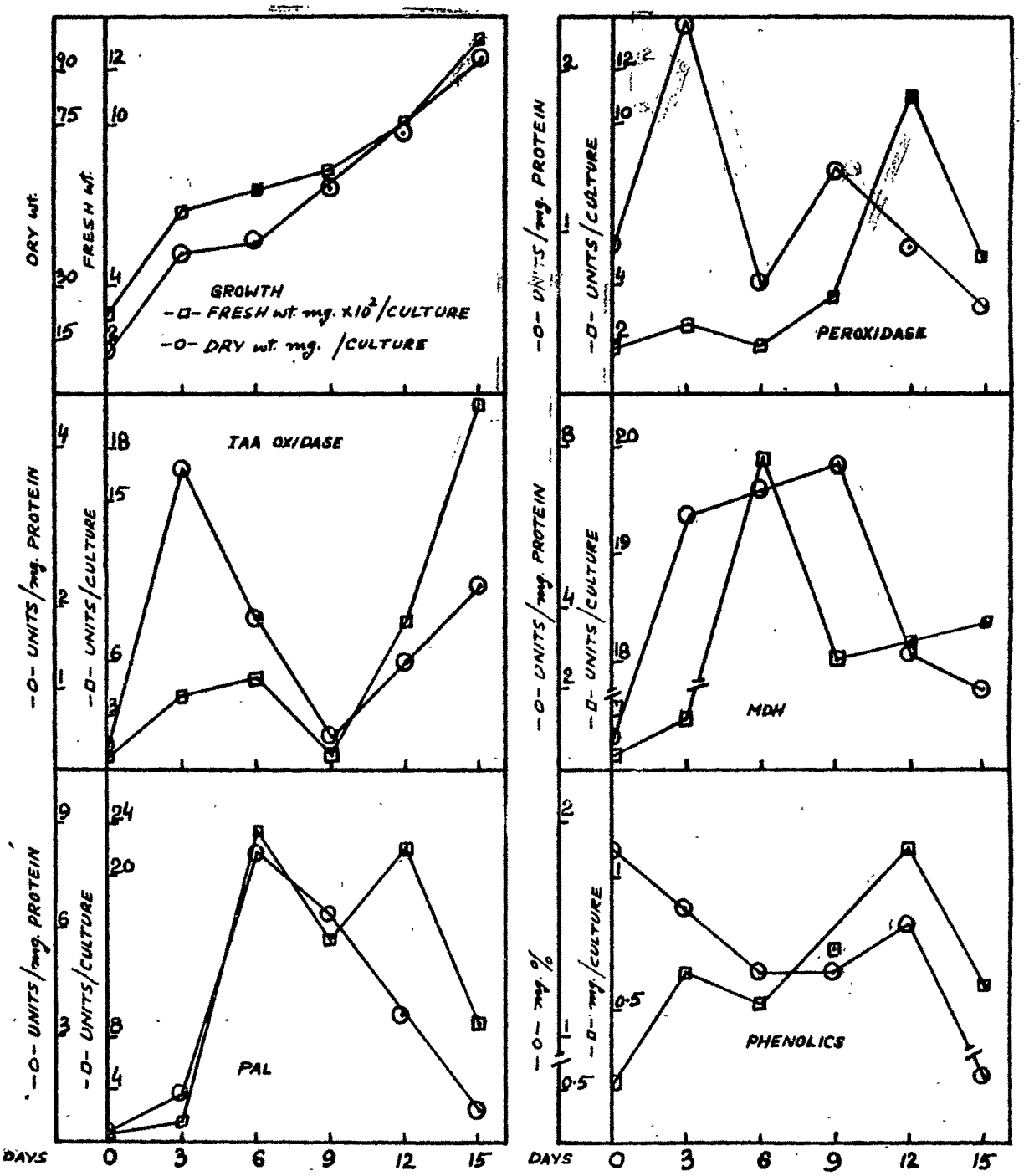


Fig. 83.

Experimental Details as in Table 21.

Table 22 : Growth, Polyphenols and progressive changes in the Activity of Peroxidase, IAA Oxidase, MDH and PAL in Diploid callus cultures of N. tabacum L. var. Anand-2 cultured on root differentiating medium containing supra-optimum level of sucrose.

Medium : MS + 2.0 mg/l IAA + 6% sucrose

Incubation : 15 days at 26±2° in continuous light

Inoculum : 300±30 mg fresh weight

Day	Fresh weight mg/cult.	Dry weight mg/cult.	PEROXIDASE		IAA OXIDASE		MDH		PAL		PHENOLICS	
			units/ cult.	units/mg protein	units/ cult.	units/mg protein	units/ cult.	units/mg protein	units/ cult.	units/mg protein	mg/ cult.	mg%
0	300 (±33)	12 (±3)	1.77	0.92	0.57	0.30	1.69	0.88	0.48	0.25	0.22	1.87
3	411.4 (±38)	31.0 (±2.6)	1.35	6.14	3.59	16.27	1.76	8.00	8.22	37.23	0.75	2.42
6	490.2 (±26.2)	40.4 (±4.6)	1.12	0.99	2.04	1.80	7.17	6.32	6.53	5.76	1.63	4.04
9	566.4 (±41.7)	47.5 (±6.3)	1.01	0.71	3.96	2.77	4.28	3.00	10.06	7.05	1.59	3.36
12	572.1 (±22.2)	50.6 (±7.1)	2.20	0.83	7.33	2.79	5.08	1.93	30.18	11.48	1.33	2.64
15	592.3 (±21.8)	52.8 (±6.9)	3.43	0.94	10.48	2.87	2.43	0.66	60.54	16.60	1.79	3.40

Figures in parenthesis indicate standard error.

Data represented is average of 5 replicates.

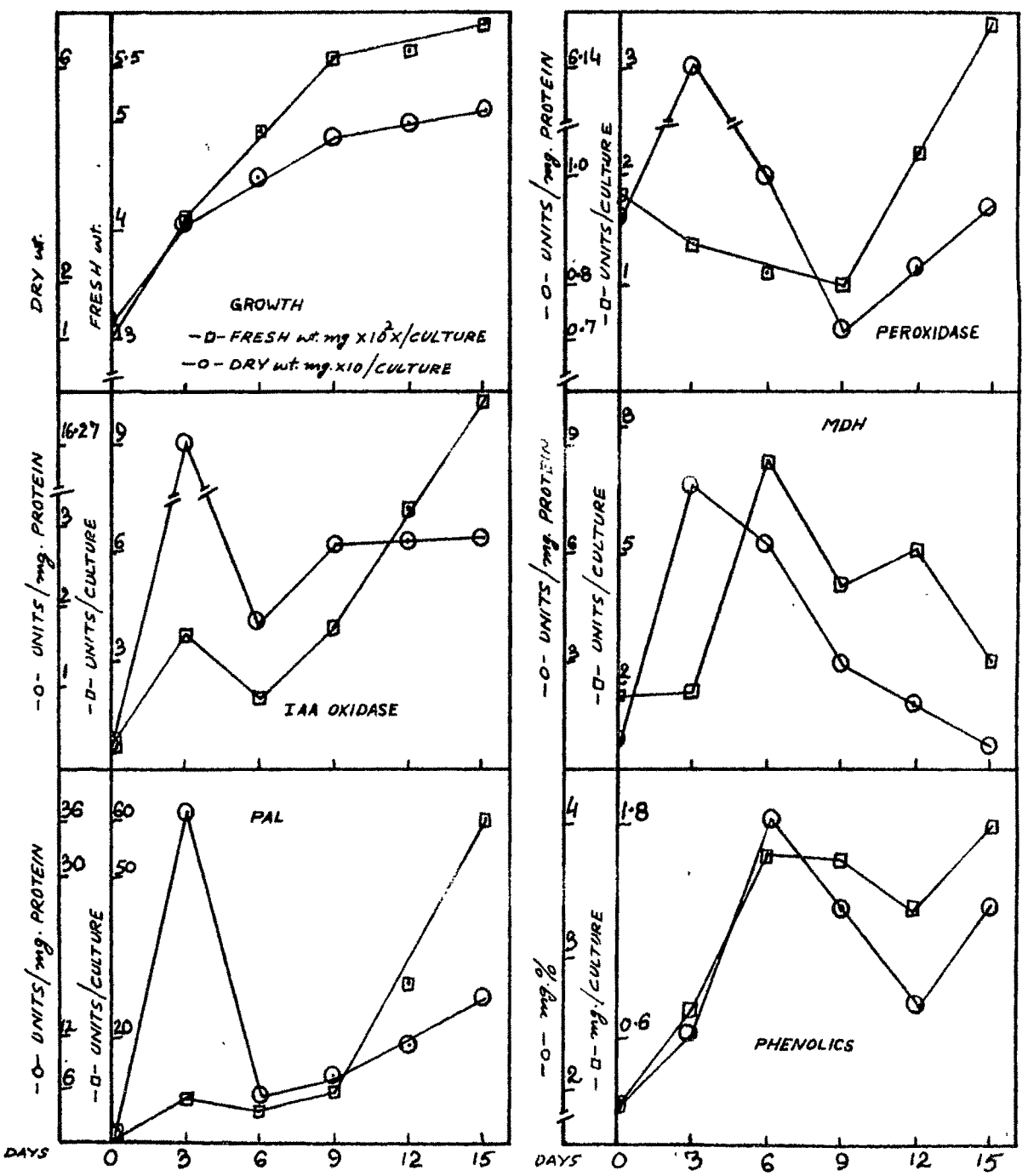


FIG. 84.

Experimental Details as in Table 22.

(c) IAA Oxidase :

Progressive changes of IAA Oxidase activity are illustrated in Figs. 83, 84 and Tables 21, 22.

The IAA Oxidase activity on Medium A increased slowly till day 6 and then dropped down by day 9. From day 9 to day 15 it increased steeply to attain its peak value. On Medium B (6% sucrose medium) total IAA Oxidase activity increased till day 3 but declined thereof till day 6. Between days 6 and 15 the enzyme activity increased almost linearly.

The specific activity of IAA Oxidase on both media attained peak value on day 3. On Medium B the activity was on decline between day 3 and day 6, climbed back by day 9 and then remained more or less steady till day 15. On the other hand, the specific activity of IAA Oxidase on Medium A decreased linearly between days 3 and 9; thenon it increased to attain its second peak value on day 15. By and large the specific activity of IAA Oxidase remained higher on Medium B than on Medium A.

On 1% sucrose containing medium 'A', no root differentiation occurred and the IAA Oxidase activity - total and specific - was on increase during the later phase of culture period during which root differentiation occurred on standard root inducing medium. On the other hand, on 6% sucrose containing

medium 'B', though total IAA Oxidase was on increase from day 6 onwards, the specific activity remained more or less steady during the period immediately preceeding and following root differentiation.

(d) MDH :

Changes of MDH activity during the culture period of 15 days of floral bud callus are illustrated in Figs. 83, 84 and Tables 21, 22.

On Medium A the total MDH activity increased rapidly and substantially between days 0 and 6. The activity declined thereof till day 9. Thereafter the activity increased linearly till day 15. On Medium B the total MDH activity increased marginally during first 3 days of culture. The peak value was attained on day 6. The activity, however, declined by day 9, but thereof increased again till day 12 before finally declining again by day 15.

The specific MDH activity on Medium A increased sharply between days 0 and 3. Between days 3 and 9 the activity increased slowly but linearly to attain the peak value. The activity between days 9 and 15, however, was on decline. On Medium B specific MDH activity increased during initial 3 days attaining its peak value. From thereon the activity declined continuously till day 15.

Both total and specific MDH activity in floral bud callus cultured on root inducing medium containing 6% sucrose (Medium B), was on decline during the days immediately preceding root differentiation. Though the same pattern of development was followed by specific MDH activity on 1% sucrose containing medium, whereon no rhizogenesis occurred, the activity was appreciably higher as compared to that in case of Medium B. The total MDH activity, on the other hand, registered peak on day 6 on both the sucrose levels - but attained an another high value on day 12 in Medium B.

(e) PAL :

The progressive changes of enzyme activity of PAL in floral bud callus during its culture period are presented in Figs. 83, 84 and Tables 21, 22.

On Medium A total PAL activity attained its peak value on day 6. The activity during this period increased nearly 50 fold. Thereof, enzyme activity declined till day 9, but thereof increased again till day 12 to attain its second peak. Between days 12 and 15 the activity was on decline again. On Medium B the enzyme activity increased nearly 17 fold by day 3 in culture. After a slight drop by day 6, the activity increased again till day 15. The increase being very sharp between days 9 and 12.

The specific activity of PAL on Medium A increased over 33 fold by day 6. Thereof, the activity decreased continuously till day 15. The specific activity of PAL on Medium B shot up nearly 150 fold by day 3. This steep rise in the activity was followed by sharp decline till day 6. From day 6 till day 15 the enzyme activity increased linearly but rather slowly.

The specific PAL activity in floral bud callus cultured on 1% sucrose medium (Medium A) was continually on the decline between days 6 and 15. On the contrary, during the same period it was on increase in callus tissues cultured on 6% sucrose medium (Medium B) on which the differentiation of roots occurred on day 12 of culture. The total activity too on Medium B showed a sharp rise before root differentiation.

(f) Phenolics :

Changes in the accumulation of phenolics in floral bud callus during the culture period of 15 days are presented in Figs. 83, 84 and Tables 21, 22.

The phenolic accumulation on Medium A (1% sucrose medium) increased between days 0 and 3 from 0.22 mg/culture to 0.64 mg/culture. The phenolic content, however, dropped to 0.52 mg/culture by day 6. Between days 6 and 12 the phenolic content increased to 1.10 mg/culture. The total accumulation of phenolics dropped to 0.61 mg/culture by day 15. On Medium B

(6% sucrose medium) the phenolic content increased from 0.22 mg/culture to 1.63 mg/culture between days 0 and 6. By day 12 the phenolic content had, however, dropped down to 1.33 mg/culture. On day 15 it had again increased upto 1.79 mg/culture. On culture basis the phenolic content was always higher in callus tissues on Medium B than on Medium A.

On percentage basis the phenolic accumulation on Medium A declined between days 0 and 6 from 1.87 mg% to 1.27 mg%. Between days 6 and 9 the percentage content of phenolics did not change. It, however, increased to 1.52 mg% by day 12, but declined appreciably to 0.65 mg% by day 15. Medium B on the whole supported higher accumulation of phenolics than did Medium A. The phenolic content in callus tissues on Medium B increased from 1.87 mg% to 4.04 mg% by day 6 in culture. A linear decline occurred in the phenolic content between days 6 and 12. On day 12 the value had declined to 2.64 mg%. By day 15 the phenolic accumulation had, however, increased again to 3.4 mg%.

On 6% sucrose containing medium (Medium B) whereon root differentiation occurred on day 12, the phenolic content was on decline during the days preceding root differentiation. Phenolic content, however, increased following root differentiation. On the contrary, on 1% sucrose medium (Medium A), whereon root differentiation was totally absent, the phenolic

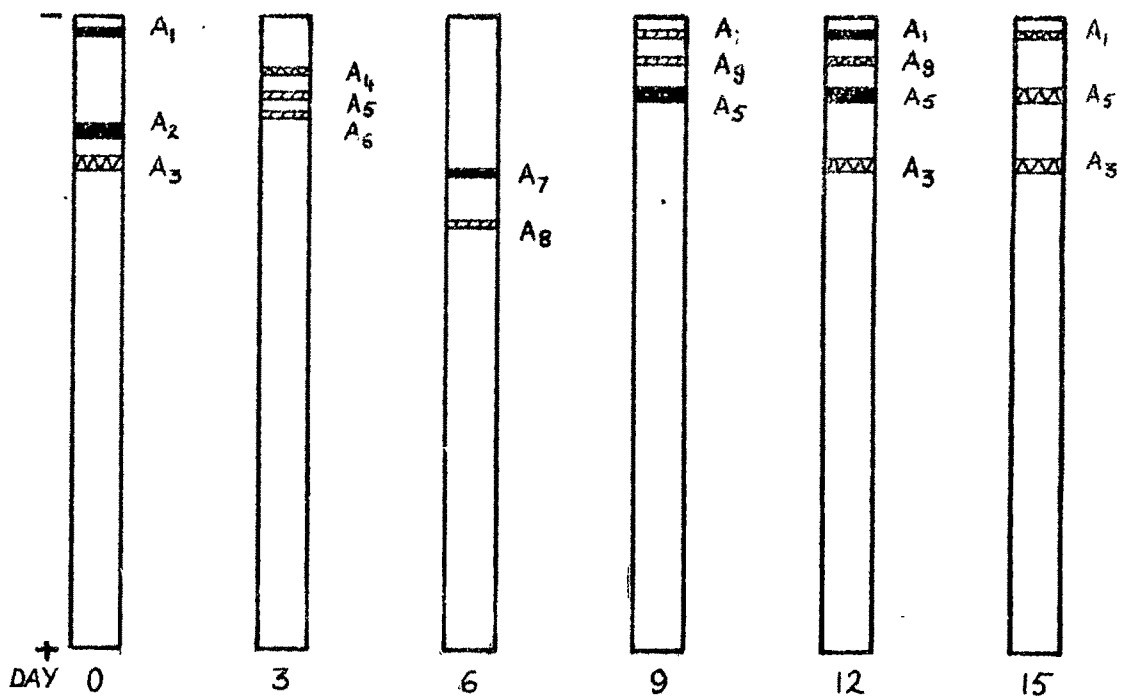
accumulation demonstrated inverse pattern of development.

(g) Peroxidase Isoenzymes :

Changes in the peroxidase isoenzyme banding pattern are illustrated in Figs. 83a and 84a.

On Medium A (1% sucrose medium) the initial three anodic isoperoxidases A_1, A_2 and A_3 were completely suppressed till day 6. The isoperoxidase A_1 reappeared on days 9, 12 and 15, whereas isoperoxidase A_3 reappeared on days 12 and 15. Isoperoxidase A_2 remained suppressed throughout the culture period. New isoperoxidases A_4, A_5 and A_6 appeared on day 3; of these A_4 and A_6 were never seen again. The isoperoxidase A_5 which was repressed on day 6, reappeared on days 9, 12 and 15. Isoperoxidase A_7 and A_8 made appearance on day 6. Peroxidase isoenzyme A_9 appeared on days 9 and 12. All new isoenzymes of peroxidase, A_4-A_9, were slow migrating. The isoperoxidase P_R associated with root initiation was conspicuously absent.

On Medium B (6% sucrose medium) of the three initial anodic isoperoxidases A_2 and A_3 were repressed for the entire culture period. A_1 was, however, synthesized on day 15 of culture. Isoperoxidase A_4 appeared on days 3, 9, 12 and 15 and A_5 on day 6. The isoperoxidase P_R appeared with the differentiation of roots on day 12.



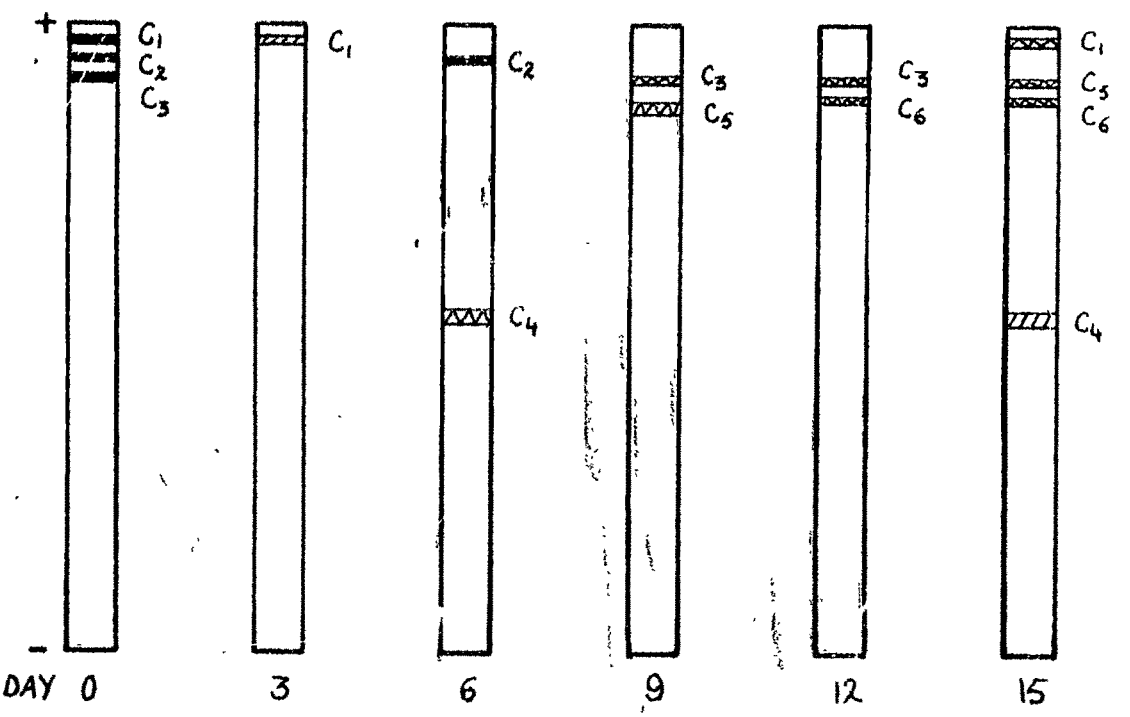


FIG. 83 a.

Anodic and Cathodic Isoperoxidase Patterns.

Experimental Details as in Table 21.

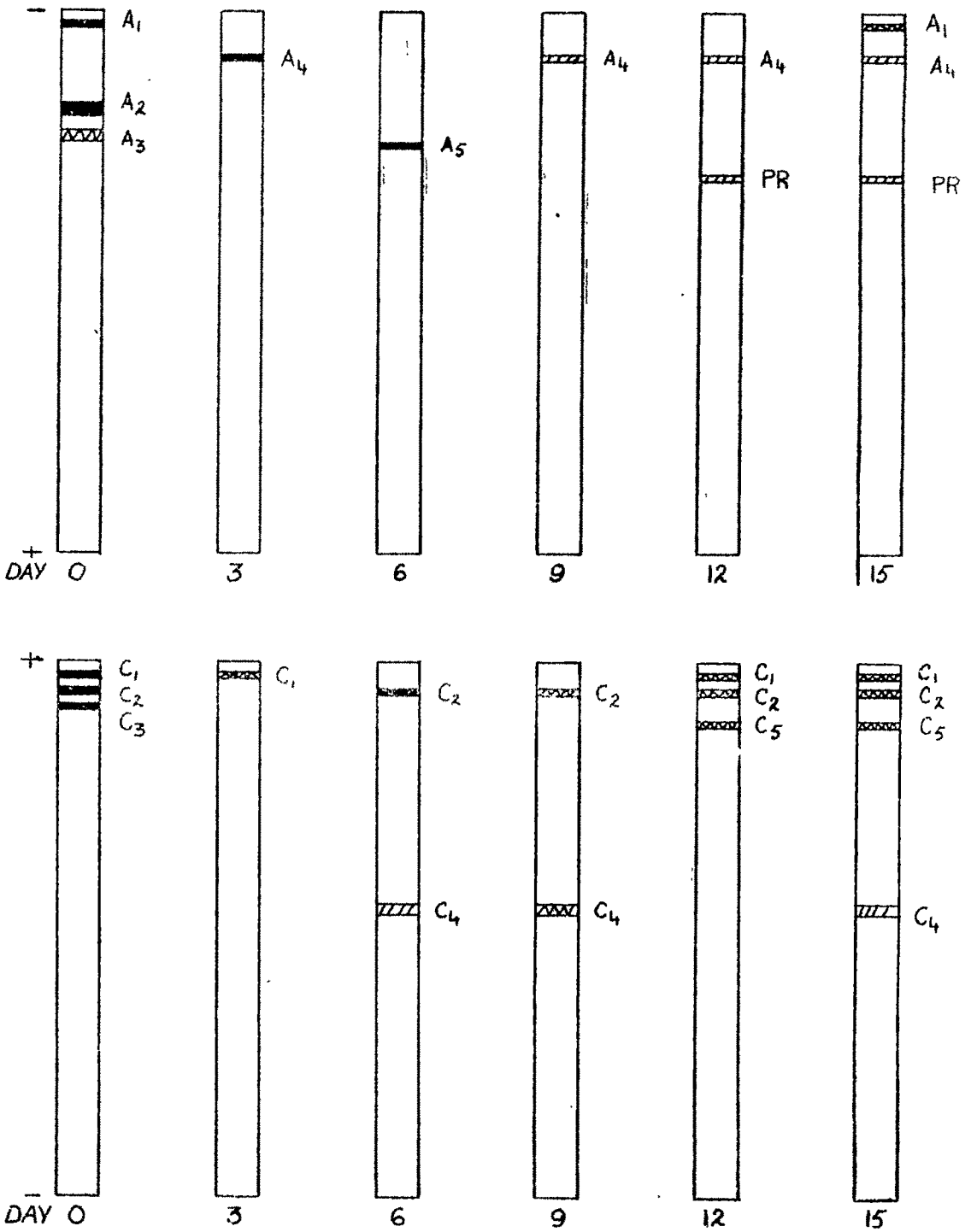


FIG. 84 a

Anodic and Cathodic Isoperoxidase Patterns.
Experimental Details as in Table 22.

On Medium A (1% sucrose medium) cathodic isoperoxidase C_1 appeared on days 0, 3 and 15, C_2 on days 0 and 6, and C_3 on days 0, 9, 12 and 15. Fast migrating cathodic isoperoxidase C_4 appeared on days 6 and 15. A slow migrating isoperoxidase C_5 was synthesized on day 9. C_6 a slow migrating cathodic isoenzyme was synthesized on days 12 and 15. On Medium B (6% sucrose medium) of the initial three cathodic isoperoxidases C_1, C_2 and C_3, the last one was completely repressed during the culture period. C_1 appeared on days 3, 12 and 15; C_2 appeared on all days except day 3. A fast migrating isoenzyme C_4 was synthesized on days 6, 9, and 15; whereas C_5 a slow migrating one appeared on days 12 and 15.

Expt. 28 : Studies with floral bud callus cultured on root inducing medium supplemented with various phenolic acids.

Healthy callus pieces weighing 300±30 mg by fresh weight were cultured on 20 ml of MS medium containing 2.0 mg/l IAA and 3% sucrose and supplemented with various phenolic acids individually. The phenolic acids and their concentrations tested were :

(a) t-Cinnamic acid (100 μM)
(b) t-Cinnamic acid (500 μM)
(c) Caffeic acid (100 μM)
(d) Caffeic acid (500 μM)
(e) Ferulic acid (100 μM)
(f) Ferulic acid (500 μM)
(g) p-Hydroxybenzoic acid (1.0 μM)
(h) p-Hydroxybenzoic acid (10.0 μM)

The culture vessels were incubated at 26±2° in continuous light. Every three days a fixed number of 5 replicates was harvested for the analysis of growth, enzymes, isoperoxidase banding pattern and phenolic accumulation.

(a) Growth :

Growth, expressed as increase in fresh and dry weights, of floral bud callus cultured on the root-inducing medium

(MS basal + 2 mg/l IAA + 3% sucrose) supplemented with phenolic acids is illustrated in Figs. 85-92 and Tables 23-30.

All the phenolic acids enhanced growth at the two different levels tested. Maximum increase in fresh weight - 6.45 fold - was recorded on medium (b) containing 500 μM t-cinnamic acid. 100 μM caffeic acid containing medium (c) gave the maximum increase in dry weight - 14.16 fold.

Addition of t-cinnamic acid at 100 (a) and 500 μM (b) brought about an increase of 5.3 and 6.45 fold in fresh weight, and 12.47 and 13.07 fold increase in dry weight respectively (Figs. 85, 86; Tables 23, 24).

Caffeic acid at 100 (c) and 500 μM (d) increased fresh weight of the callus tissues by 6.29 and 5.09 fold, and the dry weight increased by 14.16 and 10.67 folds respectively (Figs. 87, 88; Tables 25, 26).

The fresh weight registered increase of 5.42 and 4.92 fold, and dry weight increased by 12.71 and 13.35 fold when floral bud callus was cultured on medium containing 100 (e) and 500 μM (f) of ferulic acid respectively (Figs. 89, 90; Tables 27, 28).

Addition of p-hydroxybenzoic acid - 1.0 (g) and 10.0 μM (h) - into the medium did not bring about as marked increase in fresh and dry weights as in case of other phenolic acids.

The corresponding increases in fresh and dry weights for the two concentrations were 3.04 and 3.21 fold in fresh weight, and 6.98 and 7.5 folds in dry weight (Figs. 91, 92; Tables 29, 30).

Of significance was the observation that though addition of phenolic acids enhanced growth, it nevertheless, suppressed differentiation of roots in all cases.

(b) Peroxidase :

The peroxidase activity in floral bud callus cultured on root inducing medium supplemented with phenolic acids is illustrated in Figs. 85-92 and Tables 23-30.

The peroxidase activity in floral bud callus on medium (a) supplemented with 100 μM t-cinnamic acid registered a slight drop in activity by day 3. During the course of culture two peaks were attained, one each on days 6 and 12 (Fig. 85, Table 23). On the other hand, the peroxidase activity with an initial drop by day 3 continued to increase till day 15 in floral bud callus cultured on medium (b) supplemented with 500 μM t-cinnamic acid (Fig. 86 and Table 24). The peak value of peroxidase was higher on the latter than on the former medium. The specific activity of peroxidase on both the media attained the peak value on day 6. On 100 μM t-cinnamic acid containing medium a lag of three days in the enzyme activity

was observed, whereas on 500 μM t-cinnamic acid containing medium the activity increased from day 0 onwards till day 15.

In callus cultured on media supplemented with 100 (c) and 500 μM (d) caffeic acid, the peroxidase activity declined by day 3, but thereof increased in both the cases to its peak value on day 12. Between days 12 and 15 the activity dropped again (Figs. 87, 88 and Tables 25, 26). The specific activity of peroxidase remained more or less steady till day 6 in the callus cultured on 100 μM caffeic acid containing medium. From thereon it increased to reach its peak value on day 9. Between days 9 and 15 the activity declined continuously (Fig. 87, Table 25). On 500 μM Caffeic acid containing medium the specific activity of peroxidase remained stationary only till day 3, from where it increased to attain its peak value on day 6. The enzyme activity, however, declined between days 6 and 15 (Fig. 88 and Table 26).

The peroxidase activity in the callus cultured on medium (e) containing 100 μM ferulic acid remained essentially stable till day 6, increased from thereon till day 9, stabilizes again between days 9 and 12 and then shot up to its peak value on day 15 (Fig. 89, Table 27). On the contrary, the peroxidase activity remained stable till day 3 in callus tissues cultured on medium (f) containing 500 μM ferulic acid. The enzyme activity attained its peak value on

day 9 and then dropped (Fig. 90, Table 28). The specific activity of peroxidase on 100 μM ferulic acid medium increased to its peak value on day 9. By day 12 it dropped to its lowest value. Thereof, however, it increased again till day 15 (Fig. 89 and Table 27). On 500 μM medium the specific activity of peroxidase declined between days 0 and 3 before attaining its peak value on day 9. The activity declined again between days 9 and 15 (Fig. 90, Table 28).

The peroxidase activity on both, 1.0 (g) and 10.0 (h) μM p-hydroxybenzoic acid, media declined by day 3 only to climb steadily thereof and attain the peak value on day 15. The peroxidase activity remained higher on the latter medium (Figs. 91, 92; Tables 29, 30). The specific activity of peroxidase on 1.0 μM p-hydroxybenzoic acid medium increased slowly between days 3 and 12. It, however, shot up to its peak value on day 15 (Fig. 91, Table 29). On 10.0 μM p-hydroxybenzoic acid containing medium the specific activity of peroxidase declined between days 0 and 3. Thereof it increased till day 9, remained steady between days 9 and 12, and finally increased again to reach the peak value on day 15 (Fig. 92, Table 30).

With the exception of p-hydroxybenzoic acid (1.0 and 10.0 μM), the addition of all other phenolic acids resulted in decline of specific peroxidase activity during the period -

Table : 23. Growth, Polyphenols and progressive changes in the Activity of Peroxidase, IAA Oxidase, MDH and PAL in Diploid callus cultures of N. tabacum L. var. Anand-2 cultured on root differentiating medium containing 100 uM t-cinnamic acid.

Medium : MS + 2.0 mg/l IAA + 100 uM t-cinnamic acid + 3% sucrose

Incubation : 15 days at 26±2° in continuous light

Inoculum : 300±30 mg fresh weight

Day	Fresh weight mg/cult.	Dry weight mg/cult.	PEROXIDASE		IAA OXIDASE		MDH		PAL		PHENOLICS	
			units/ cult.	units/mg protein	units/ cult.	units/mg protein	units/ cult.	units/mg protein	units/ cult.	units/mg protein	mg/ cult.	mg%
0	300 (±30)	12 (±3)	1.77	0.92	0.57	0.30	1.69	0.88	0.48	0.25	0.22	1.87
3	522.0 (±27)	22.2 (±2.4)	1.73	0.89	1.08	0.55	6.26	3.21	12.75	6.55	0.67	3.06
6	952.7 (±48.2)	69.2 (±3.6)	6.11	1.86	13.54	4.11	12.19	3.70	2.11	0.64	1.98	2.74
9	1150.0 (±52.6)	83.6 (±7.8)	4.09	0.96	10.03	2.36	3.68	0.86	8.93	2.11	3.47	4.16
12	1380.5 (±28.7)	129.9 (±4.1)	6.98	0.50	1.51	0.10	10.06	0.72	58.70	4.21	5.68	4.38
15	1591.4 (±23.2)	149.7 (±8.9)	6.60	0.32	2.03	0.10	29.65	1.46	60.82	3.00	4.52	3.02

Figures in parenthesis indicate standard error.

Data represented is average of 5 replicates.

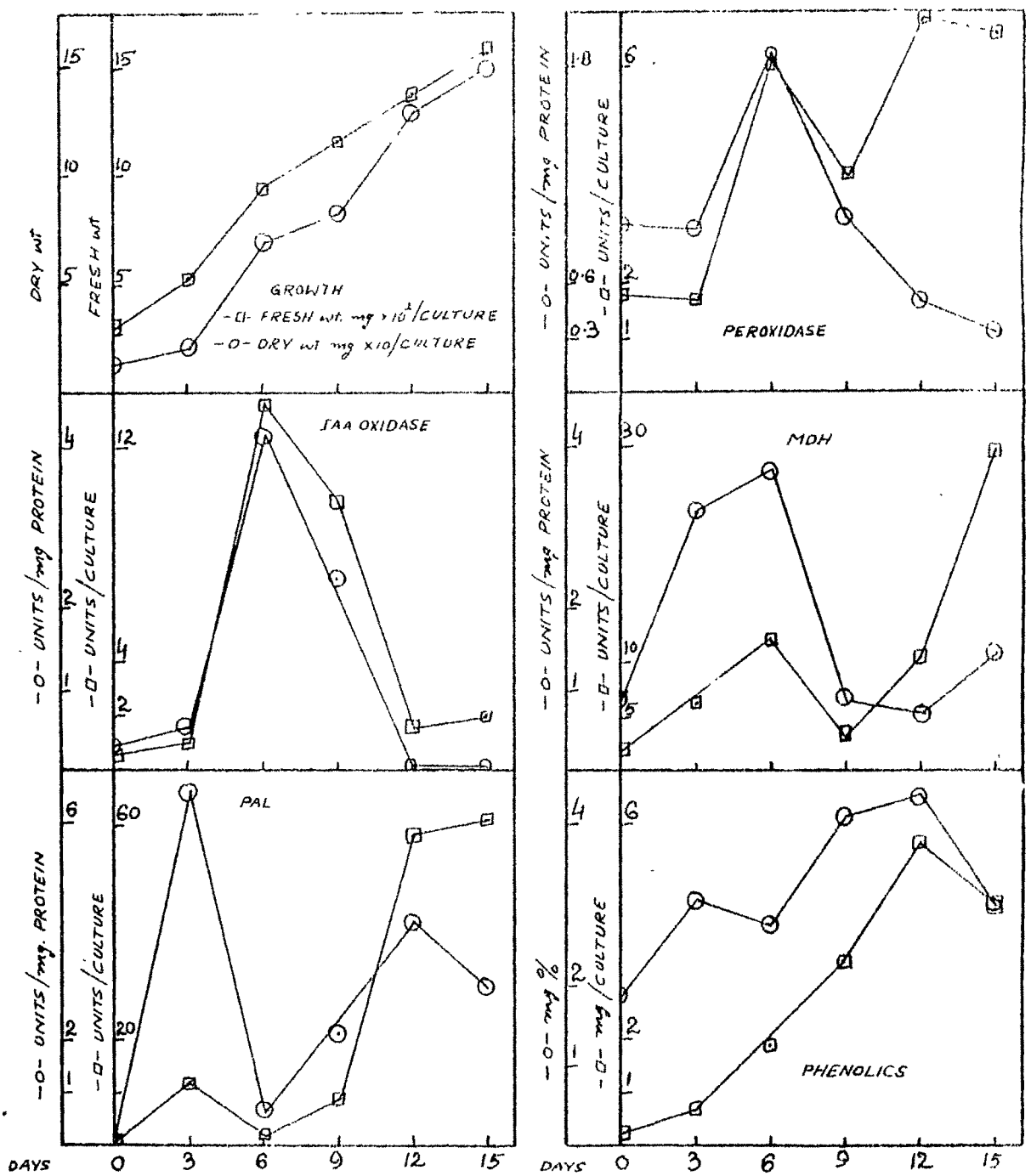


FIG. 85

Experimental Details as in Table 23.

Table : 24. Growth, Polyphenols and progressive changes in the Activity of Peroxidase, IAA Oxidase, MDH and PAL in Diploid callus cultures of N. tabacum L. var Anand-2 cultured on root differentiating medium containing 500 uM t-cinnamic acid.

Medium : MS + 2.0 mg/l IAA + 500 uM t-cinnamic acid +3% sucrose

Incubation : 15 days at 26±2° in continuous light

Inoculum : 300±30 mg fresh weight

Day	Fresh weight mg/cult.	Dry weight mg/cult.	PEROXIDASE		IAA OXIDASE		MDH		PAL		PHENOLICS	
			units/ cult.	units/mg protein	units/ cult.	units/mg protein	units/ cult.	units/mg protein	units/ cult.	units/mg protein	mg/ cult.	mg%
0	300 (±30)	12 (±3)	1.77	0.92	0.57	0.30	1.69	0.88	0.48	0.25	0.22	1.87
3	471.2 (±41.2)	26.8 (±4.0)	1.43	1.23	0.98	0.84	5.84	5.05	2.09	1.80	0.77	2.91
6	751.2 (±23.8)	64.8 (±6.1)	2.37	1.69	7.79	5.56	7.81	5.58	11.68	8.34	2.47	3.82
9	1007.3 (±81.6)	86.8 (±5.5)	3.94	0.72	25.48	4.67	10.47	1.92	2.23	0.41	3.85	4.44
12	1604.6 (±72.2)	130.0 (±10.1)	7.83	0.69	5.04	0.44	14.46	1.27	4.43	0.39	3.67	2.83
15	1937.4 (±38.1)	156.9 (±12.6)	9.88	0.57	5.16	0.29	41.84	2.42	5.73	0.33	3.79	2.42

Figures in parenthesis indicate standard error.
Data represented is average of 5 replicates.

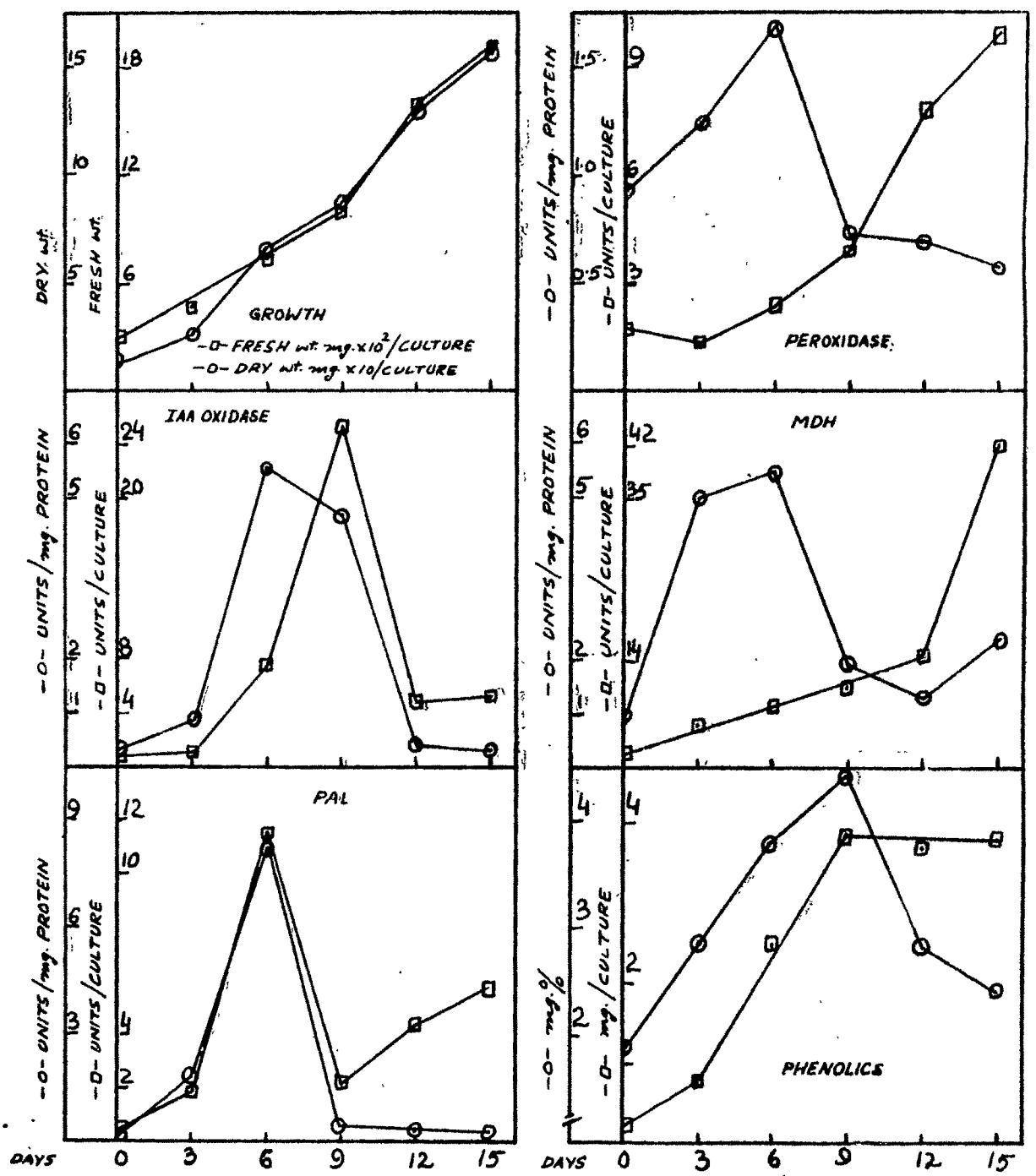


FIG. 86.

Experimental Details as in Table 24.

Table : 25. Growth, Polyphenols and progressive changes in the Activity of Peroxidase, IAA Oxidase, MDH and PAL in Diploid callus cultures of N. tabacum L. var anand-2 cultured on root differentiating medium containing 100 uM caffeic acid.

Medium : MS + 2.0 mg/l IAA + 100 uM caffeic acid + 3% sucrose

Incubation : 15 days at 26±2° in continuous light

Inoculum : 300±30 mg fresh weight

Day	Fresh weight mg/cult.	Dry weight mg/cult.	PEROXIDASE		IAA OXIDASE		MDH		PAL		PHENOLICS	
			units/ cult.	units/mg protein	units/ cult.	units/mg protein	units/ cult.	units/mg protein	units/ cult.	units/mg protein	mg/ cult.	mg%
0	300 (±30)	12 (±3)	1.77	0.92	0.57	0.30	1.69	0.88	0.48	0.25	0.22	1.87
3	501.5 (±40)	32.2 (±5.8)	1.43	0.98	1.28	0.87	8.02	5.50	1.11	0.76	0.73	2.28
6	801.8 (±24)	57.2 (±7.6)	2.98	0.95	11.40	3.63	6.41	2.04	5.33	1.70	1.39	2.44
9	1013.9 (±81.6)	72.3 (±4.5)	3.38	1.17	2.87	1.00	3.65	1.26	14.64	5.08	1.89	2.62
12	1584.2 (±72.3)	142.6 (±10.9)	6.05	0.77	0.32	0.04	49.21	6.32	23.54	3.02	4.09	2.87
15	1889.6 (±90.7)	170.0 (±12.8)	5.77	0.53	0.42	0.03	33.49	3.09	17.68	1.64	4.99	2.94

Figures in parenthesis indicate standard error.

Data represented is average of 5 replicates.

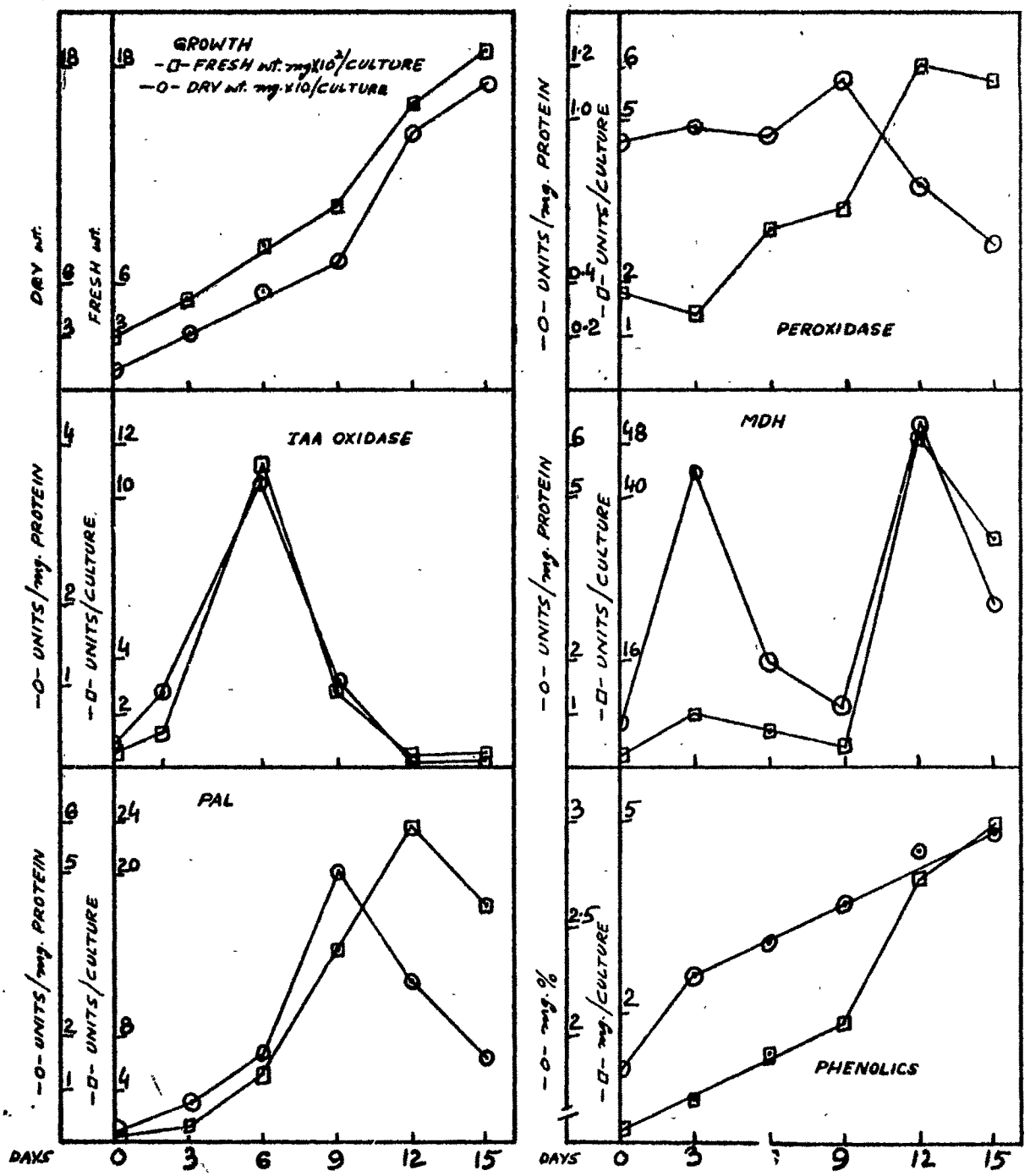


Fig. 87.

Experimental Details as in Table 25.

Table : 26. Growth, Polyphenols and progressive changes in the Activity of Peroxidase, IAA Oxidase, MDH and PAL in Diploid callus cultures of N. tabacum L. var Anand-2 cultured on root differentiating medium containing 500 uM caffeic acid.

Medium : MS + 2.0 mg/l IAA + 500 uM caffeic acid + 3% sucrose

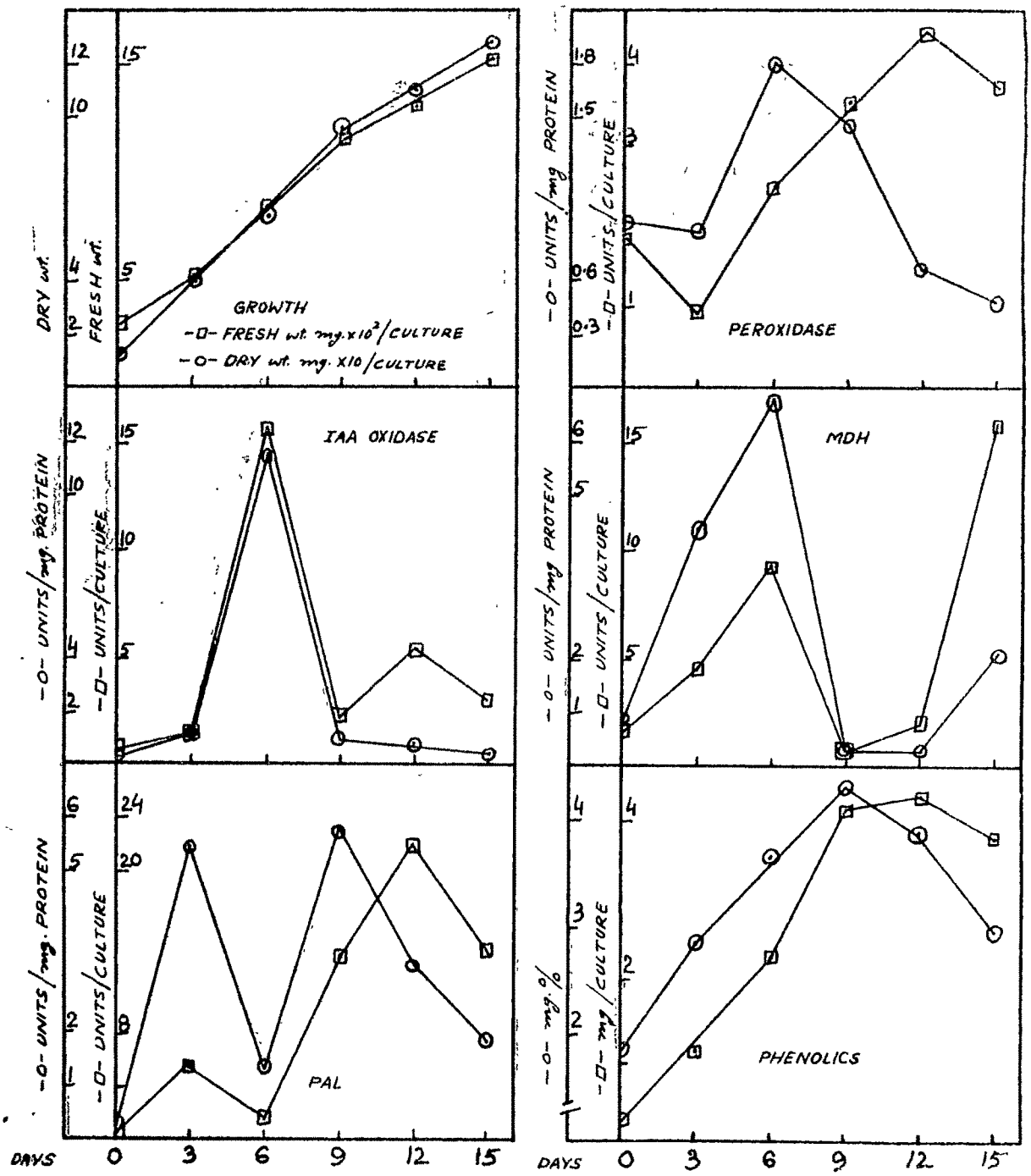
Incubation : 15 days at 26±2° in continuous light

Inoculum : 300±30 mg fresh weight

Day	Fresh weight mg/cult.	Dry weight mg/cult.	PEROXIDASE		IAA OXIDASE		MDH		PAL		PHENOLICS	
			units/ cult.	units/mg protein	units/ cult.	units/mg protein	units/ cult.	units/mg protein	units/ cult.	units/mg protein	mg/ cult.	mg%
0	300 (±30)	12 (±3)	1.77	0.92	0.57	0.30	1.69	0.88	0.48	0.25	0.22	1.87
3	530.9 (±28.9)	40.0 (±2.8)	0.94	0.89	1.35	1.27	4.67	4.40	5.89	5.55	1.14	2.85
6	835.0 (±42.3)	63.9 (±4.6)	2.48	1.82	15.81	11.57	9.35	6.84	1.85	1.35	2.31	3.63
9	1145.8 (±81.7)	96.1 (±7.2)	3.55	1.48	2.39	1.00	0.91	0.38	14.00	5.84	4.13	4.30
12	1316.3 (±76.6)	110.4 (±8.6)	4.42	0.64	5.61	0.82	2.13	0.31	22.36	3.27	4.27	3.87
15	1528.1 (±96.8)	128.1 (±4.6)	3.74	0.49	3.12	0.41	15.81	2.08	14.16	1.86	3.79	2.96

Figures in parenthesis indicate standard error.

Data represented is average of 5 replicates.



Experimental Details as in Table 26.

FIG. 88

Table : 27. Growth, Polyphenols and progressive changes in the Activity of Peroxidase, IAA Oxidase, MDH and PAL in Diploid callus cultures of N. tabacum L. var Anand-2 cultured on root differentiating medium containing 100 uM Ferulic acid.

Medium : MS + 2.0 mg/l IAA + 100 uM Ferulic acid + 3% sucrose

Incubation : 15 days at 26±2° in continuous light

Inoculum : 300±30 mg fresh weight

Day	Fresh weight mg/cult.	Dry weight mg/cult.	PEROXIDASE		IAA OXIDASE		MDH		PAL		PHENOLICS	
			units/ cult.	units/mg protein	units/ cult.	units/mg protein	units/ cult.	units/mg protein	units/ cult.	units/mg protein	mg/ cult.	mg%
0	300 (±30)	12 (±3)	1.77	0.92	0.57	0.30	1.69	0.88	0.48	0.25	0.22	1.87
3	525.0 (±43.6)	26.2 (±4)	1.61	1.54	2.97	1.97	5.04	4.80	4.66	4.44	0.89	3.42
6	679.2 (±28.2)	48.2 (±3.1)	1.73	2.33	10.49	14.13	3.72	5.01	2.26	3.04	1.37	2.86
9	937.1 (±62.5)	66.6 (±4.3)	4.94	3.62	13.99	10.26	3.74	2.75	6.24	4.57	2.31	3.48
12	1306.1 (±81.4)	122.6 (±7.9)	4.91	0.72	4.10	0.60	11.29	1.66	22.13	3.27	1.61	1.32
15	1626.3 (±76.7)	152.6 (±10.4)	13.80	1.34	12.79	1.24	26.67	2.59	15.82	1.53	2.35	1.54

Figures in parenthesis indicate standard error.

Data represented is average of 5 replicates.

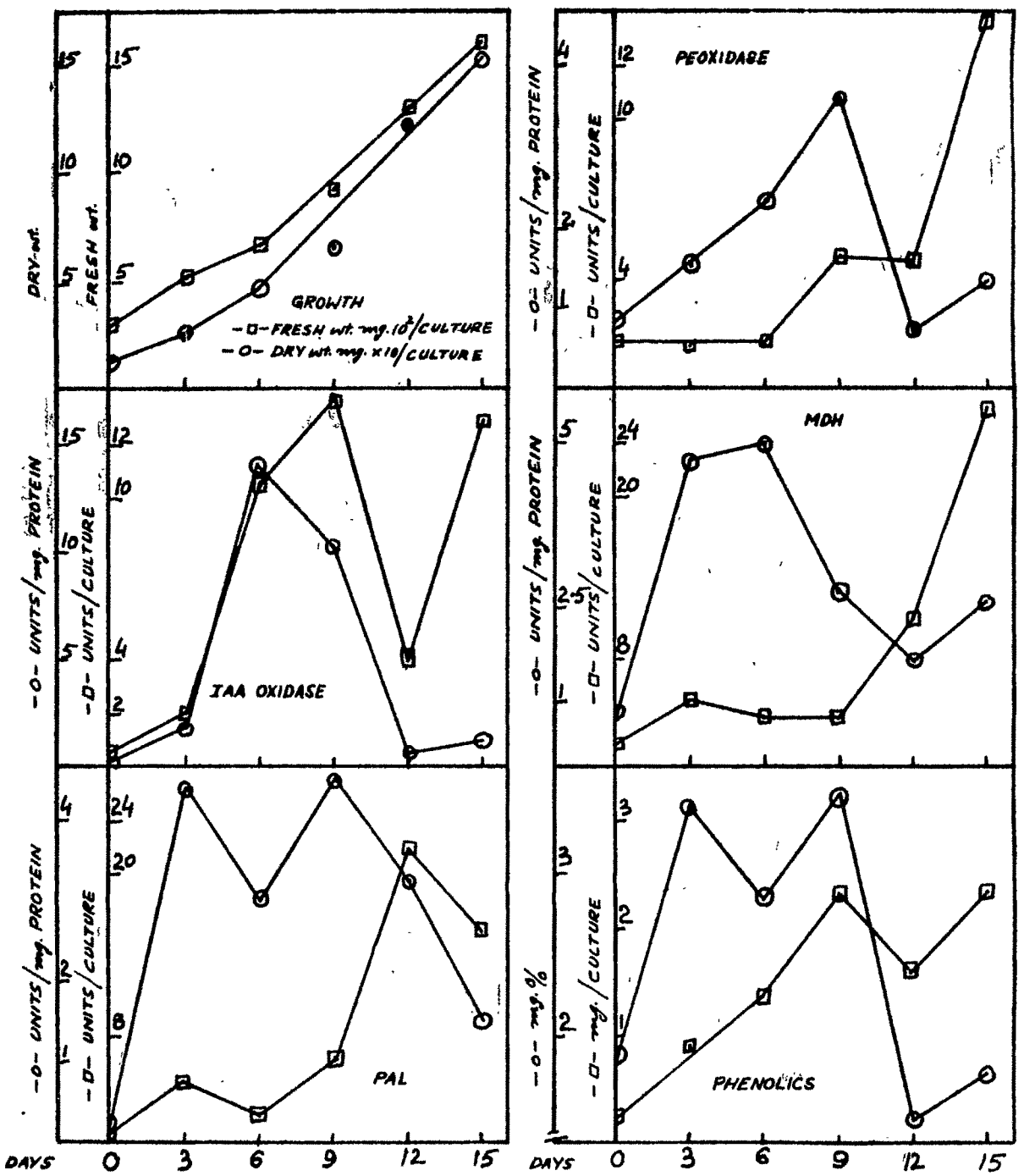


FIG. 89.

Experimental Details as in Table 27.

Table : 28. Growth, Polyphenols and progressive changes in the Activity of Peroxidase, IAA Oxidase, MDH and PAL in Diploid callus cultures of N. tabacum L. var Anand-2 cultured on root differentiating medium containing 500 uM Ferulic acid.

Medium : MS + 2.0 mg/l IAA + 500 uM Ferulic acid + 3% sucrose

Incubation : 15 days at 26±2° in continuous light

Inoculum : 300±30 mg fresh tissue

Day	Fresh weight mg/cult.	Dry weight mg/cult.	PEROXIDASE		IAA OXIDASE		MDH		PAL		PHENOLICS	
			units/ cult.	units/mg protein	units/ cult.	units/mg protein	units/ cult.	units/mg protein	units/ cult.	units/mg protein	mg/ cult.	mg%
0	300 (±30)	12 (±3)	1.77	0.92	0.57	0.30	1.69	0.88	0.48	0.25	0.22	1.87
3	515.9 (±18.1)	29.1 (±4.8)	1.58	0.55	2.68	0.93	8.25	2.88	2.29	0.80	0.70	2.43
6	686.8 (±42.3)	47.3 (±5.7)	6.18	2.32	5.45	2.05	5.54	2.08	5.33	2.00	1.36	2.88
9	872.4 (±66.6)	60.1 (±6.0)	24.31	6.32	20.63	5.36	1.12	0.21	3.87	1.00	2.01	3.36
12	1285.9 (±71.8)	139.4 (±8.2)	7.20	0.79	8.99	0.99	1.03	0.11	8.23	0.90	3.65	2.62
15	1478.6 (±89.5)	160.2 (±12.7)	6.12	0.43	22.92	1.63	1.38	0.09	11.37	0.81	3.39	2.12

Figures in parenthesis indicate standard error.

Data represented is average of 5 replicates.

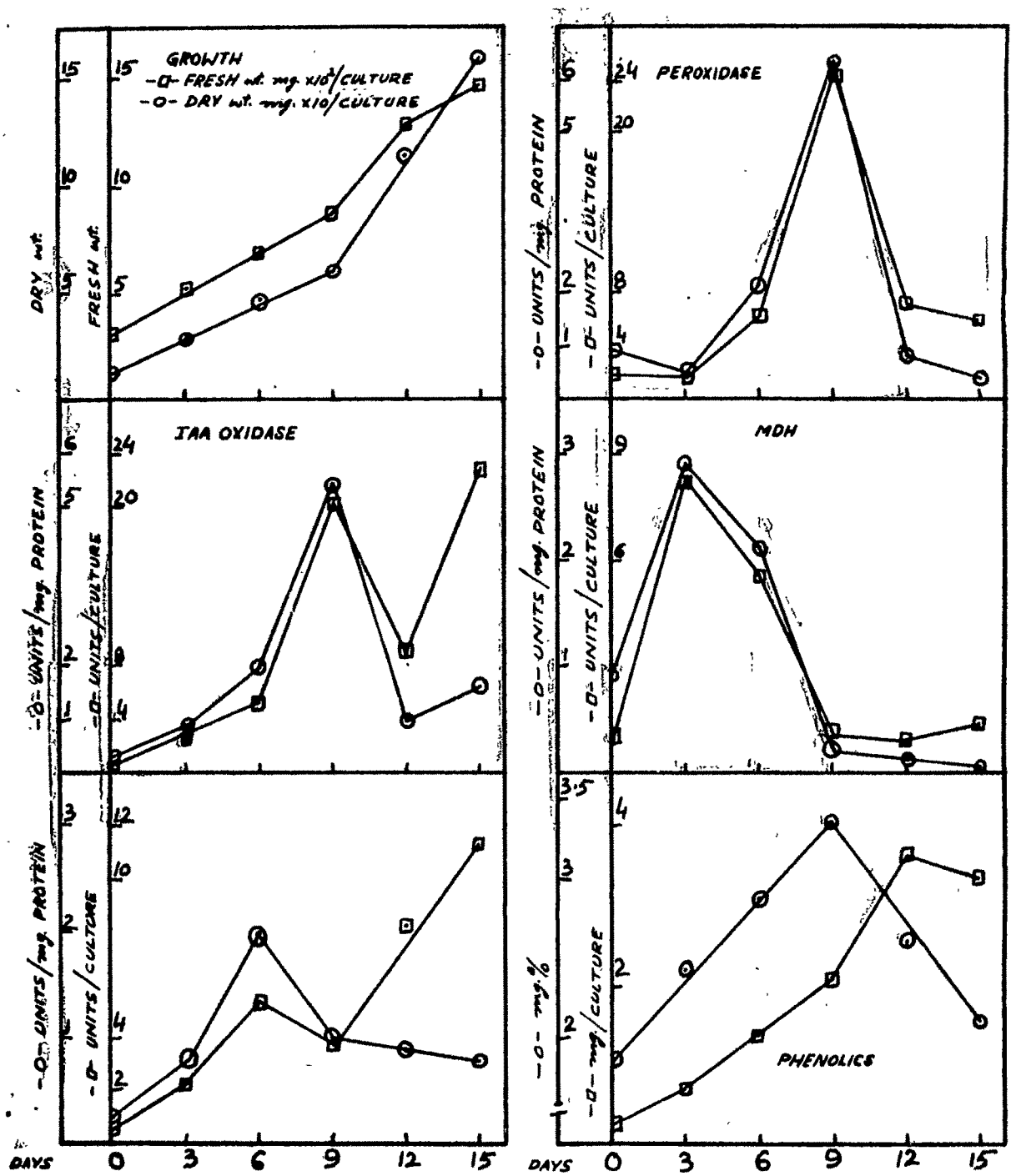


FIG. 90.

Experimental Details as in Table 28

Table : 29. Growth, Polyphenols and progressive changes in the Activity of Peroxidase, IAA Oxidase, MDH and PAL in Diploid callus cultures of N. tabacum L. var Anand-2 cultured on root differentiating medium containing 1.0 uM p-Hydroxybenzoic acid.

Medium : MS + 2.0 mg/l IAA + 1.0 uM p-Hydroxybenzoic acid + 3% sucrose

Incubation : 15 days at 26±2° in continuous light

Inoculum : 300±30 mg fresh tissue

Day	Fresh weight mg/cult.	Dry weight mg/cult.	PEROXIDASE		IAA OXIDASE		MDH		PAL		PHENOLICS	
			units/ cult.	units/mg protein	units/ cult.	units/mg protein	units/ cult.	units/mg protein	units/ cult.	units/mg protein	mg/ cult.	mg%
0	300 (±30)	30 (±3)	1.77	0.92	0.57	0.30	1.69	0.88	0.48	0.25	0.22	1.87
3	384.0 (±22)	18.2 (±2.9)	0.41	0.44	1.03	1.09	0.30	0.32	1.70	1.80	0.44	2.43
6	509.8 (±47.1)	40.2 (±4.8)	0.61	0.73	1.99	2.36	3.97	4.71	1.13	1.34	1.06	2.65
9	679.4 (±63.3)	53.7 (±3.3)	2.94	0.93	13.29	4.22	2.71	0.86	9.05	2.87	1.66	3.10
12	809.5 (±51.4)	47.3 (±5.0)	3.43	1.08	9.70	3.06	20.00	6.32	5.48	1.73	2.79	3.76
15	913.3 (±82.9)	83.8 (±8.5)	6.50	2.43	4.50	1.68	18.34	6.88	2.67	1.00	3.21	3.84

Figures in parenthesis indicate standard error.

Data represented is average of 5 replicates.

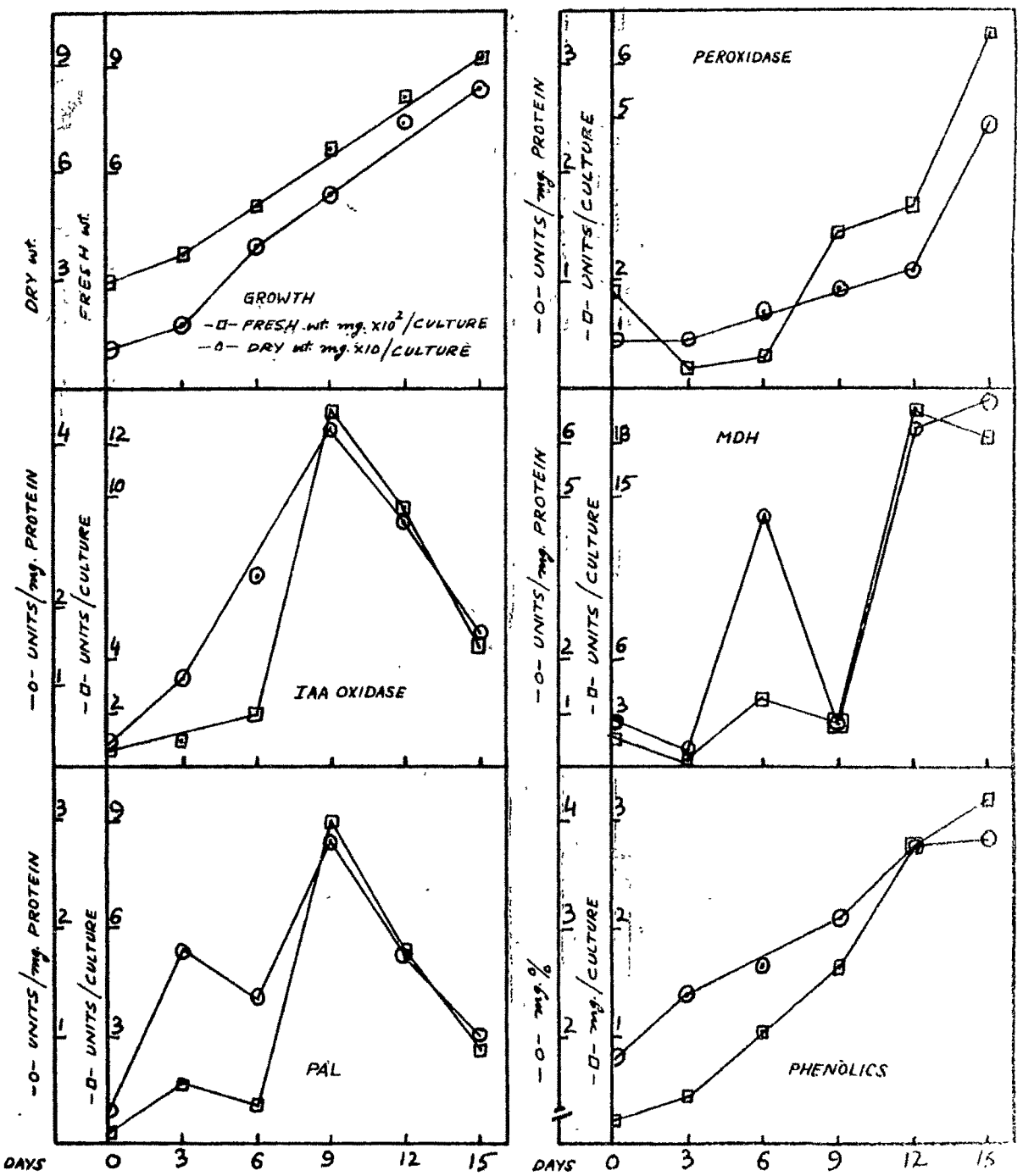


FIG. 91.

Experimental Details as in Table 29.

Table : 30. Growth, Polyphenols and progressive changes in the Activity of Peroxidase, IAA Oxidase, MDH and PAL in Diploid callus cultures of N. tabacum L. var. Anand-2 cultured on root differentiating medium containing 10.0 uM p-Hydroxybenzoic acid.

Medium : MS + 2.0 mg/l IAA + 1.0 uM p-Hydroxybenzoic acid + 3% sucrose

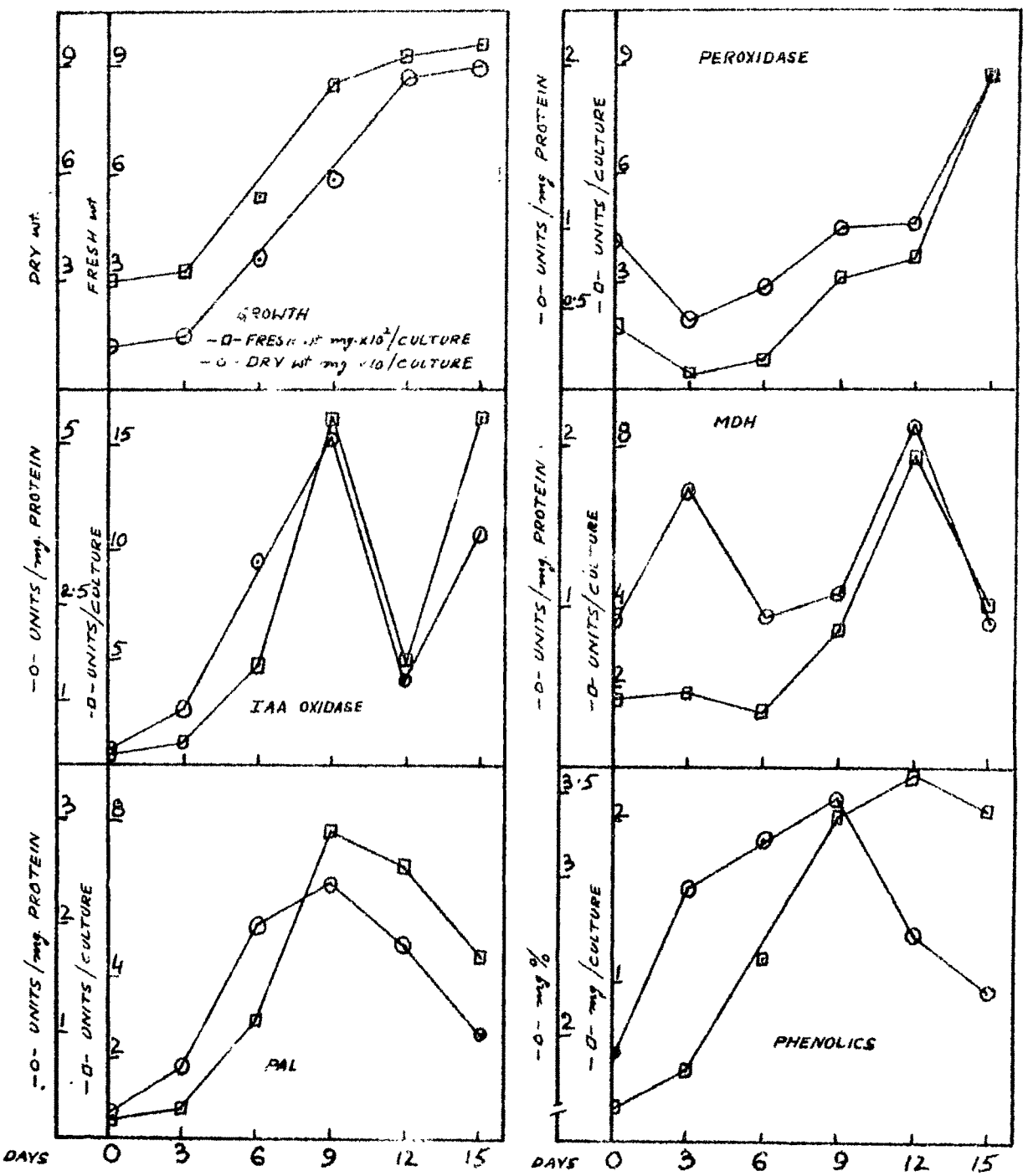
Incubation : 15 days at 26± 2° in continuous light

Inoculum : 300±30 mg fresh tissue

Day	Fresh weight mg/cult.	Dry weight mg/cult.	PEROXIDASE		IAA OXIDASE		MDH		PAL		PHENOLICS	
			units/ cult.	units/mg protein	units/ cult.	units/mg protein	units/ cult.	units/mg protein	units/ cult.	units/mg protein	mg/ cult.	mg%
0	300 (±30)	12 (±3)	1.77	0.92	0.57	0.30	1.69	0.88	0.48	0.25	0.22	1.87
3	332.2 (±12.3)	15.6 (±3.3)	0.47	0.44	0.94	0.87	1.86	1.71	0.73	0.67	0.45	1.91
6	543.7 (±50.0)	37.0 (±4.1)	0.94	0.63	4.77	3.20	1.41	0.94	3.01	2.02	1.18	3.21
9	863.2 (±41.2)	58.8 (±2.2)	3.17	0.99	16.34	5.14	3.45	1.08	7.66	2.41	2.04	3.48
12	934.9 (±76.8)	87.5 (±6.0)	3.72	1.01	4.77	1.30	7.76	2.12	6.81	1.86	2.30	2.63
15	963.6 (±88.9)	90.1 (±7.4)	8.68	1.93	16.24	3.61	4.07	0.90	4.55	1.01	2.06	2.29

Figures in parenthesis indicate standard error.

Data represented is average of 5 replicates.



Experimental Details as in Table 30.

FIG. 92.

day 6 to 15 - preceding root differentiation; though root differentiation did not occur with the addition of any of the phenolic acids tested.

(c) IAA Oxidase :

The progressive changes in the activity of IAA Oxidase in floral bud callus cultured on root inducing medium supplemented with different phenolic acids individually are illustrated in Figs. 85-92 and Tables 23-30.

Both total and specific activities of IAA Oxidase in floral bud callus cultured on medium (d) supplemented with 100 µM t-cinnamic acid followed identical developmental pattern. The enzyme activity grew from 0 day 0 till day 6 attaining its peak value. The activity dropped thereafter (Fig. 85, Table 23). On 500 µM t-cinnamic acid containing medium (b) the peak value of total IAA Oxidase activity was attained on day 9; whereas the specific activity reached its peak value on day 6 (Fig. 86, Table 24).

On 100 µM caffeic acid medium (c) the total and specific activities of IAA Oxidase increased till day 6, to reach its peak value and then decline from thereon till day 15 (Fig. 87, Table 25). The total IAA Oxidase activity on 500 µM caffeic acid medium (d) exhibited double peaked developmental pattern, one each on days 6 and 12. The specific activity, however,

showed only one peak value on day 6. In both the cases the enzyme activity declined after attaining its peak value (Fig. 88, Table 26).

The total IAA Oxidase activity achieved two peak values, one each on days 9 and 15, on both the media (e, f) containing 100 and 500 μM ferulic acid. The specific activity, however, attained peak value on day 6 on 100 μM ferulic acid medium. On 500 μM ferulic acid medium the peak value was attained on day 9. In both cases the specific activity after declining till day 12 increased marginally by day 15 (Figs. 89, 90; Tables 27, 28).

The floral bud callus tissues cultured on medium (g) containing 1.0 μM p-hydroxybenzoic acid had IAA Oxidase activity increasing, both total and specific, till day 9. Thereon the activity declined sharply till day 15 (Fig. 91 and Table 29). The total and specific IAA Oxidase activity on 10.0 μM p-hydroxybenzoic acid medium (h) demonstrated two peaks, one each on days 9 and 15 (Fig. 92, Table 30).

Incorporation of t-cinnamic acid (100, 500 μM), caffeic acid (100, 500 μM) and ferulic acid (100 μM) into the root inducing medium resulted in sharp inhibition of specific IAA Oxidase activity from day 6 onwards. A comparable trend was followed with the incorporation of p-hydroxybenzoic acid (1.0 μM) into the medium. However, in this case the inhibition

occurred from day 9 onwards. On ferulic acid (500 μM) and p-hydroxybenzoic acid (10.0 μM) containing medium there was sharp decline in the activity between days 9 and 12.

(d) MDH : Progressive changes of MDH activity in floral bud callus cultured on root inducing medium supplemented with phenolic acids individually, are presented in Figs. 85-92 and Tables 23-30.

The MDH activity on medium (a) containing 100 μM t-cinnamic acid attained two peaks values, one each on days 6 and 15. Same developmental pattern was followed by specific activity of the enzyme (Fig. 85, Table 23). On 500 μM t-cinnamic acid containing medium (b) the MDH activity increased slowly and linearly till day 12. A sharp increase was registered between days 12 and 15. The specific activity of the enzyme, however, as in the previous instance reached two peaks, one each on days 6 and 15 (Fig. 86, Table 24).

On the medium (c) supplemented with 100 μM caffeic acid, total MDH activity increased till day 3. Between days 3 and 9 the activity dropped along a linear gradient. By day 12, however, the enzyme activity increased sharply, but declined again by day 15. The specific activity of MDH on this medium followed the pattern as defined by total activity of the enzyme (Fig. 87, Table 25). The total and specific MDH activity

Incorporation of individual phenolic acids, at both the levels tested, into the root inducing medium brought about decline of PAL activity per unit protein in floral bud callus between days 12 and 15. During the same period in culture, the PAL activity per unit protein declined on standard root inducing medium and was followed by root differentiation from callus tissue. Since on the root inducing medium and also on the same medium supplemented with various phenolic acids PAL activity was on decline between days 12 and 15, no correlation could be drawn between PAL activity and root differentiation.

(f) Phenolics :

The progressive changes in the phenolic content of floral bud callus cultured on root inducing medium supplemented with different phenolic acids are illustrated in Figs. 85-92 and Tables 23-30.

In callus cultured on 100 µM t-cinnamic acid medium (a) the phenolic content increased from 0.22 mg/culture to its peak value of 5.68 mg/culture on day 12. On percentage basis two peaks were obtained. During the first phase of phenolic accumulation, it increased from 1.87 mg% to 3.06 mg% by day 3. The second peak of 4.38 mg% was obtained on day 12 (Fig. 85, Table 23).

On 500 μM t-cinnamic acid medium (b) the peak value of phenolic accumulation of 3.85 mg/culture was attained on day 9. The accumulation of phenolics fluctuated between days 9 and 15. On percentage basis also the peak value (4.44 mg%) was attained on day 9. Thereof till day 15 the phenolic content declined to 2.42 mg% (Fig. 86, Table 24).

The accumulation of phenolics in the floral bud callus cultured on 100 μM caffeic acid medium (c) increased steadily and linearly till day 9. Between days 9 and 15 the increase was sharp. During the entire culture period the phenolic accumulation rose from 0.22 mg/culture on day 0 to 4.99 mg/culture on day 15. On percentage basis also the peak value was attained on day 15 of culture, rising from 1.87 mg% to 2.94 mg% (Fig. 87, Table 25).

On culture basis the phenolic content in callus on 500 μM caffeic acid containing medium (d) increased from 0.22 mg/culture to 4.27 mg/culture by day 12. On percentage basis the peak value was, however, attained on day 9, the rise being from 1.87 mg% to 4.3 mg% (Fig. 88, Table 26).

On 100 μM ferulic acid medium (e) two peak values of 2.31 mg/culture and 2.35 mg/culture were obtained on days 9 and 15 respectively. On percentage basis also the phenolic accumulation revealed two peak values of 3.42 mg% and 3.48 mg% on days 3 and 9 respectively (Fig. 89, Table 27).

On 500 μM ferulic acid containing medium (f) the peak value for phenolic accumulation on culture basis was attained on day 12, the value being 3.65 mg/culture. On percentage basis, the peak value of 3.36 mg% was, however, attained on day 9 (Fig. 90, Table 28).

The accumulation of phenolics on both per culture and percentage basis increased till day 15 when callus was cultured on 1.0 μM p-hydroxybenzoic acid containing medium(g). The peak values attained on day 15 were 3.21 mg/culture and 3.84 mg% (Fig. 91, Table 29).

On 10.0 μM p-hydroxybenzoic acid medium (h) the peak phenolic value (2.3 mg/culture) on culture basis in callus was reached on day 12. On percentage basis the peak value of 3.48 mg% was, however, reached on day 9 (Fig. 92, Table 30).

With the exception of 100 μM caffeic acid and 1.0 μM p-hydroxybenzoic acid, addition of all other phenolic acids into the root inducing medium brought about decline of phenolic content in floral bud callus during days 12 and 15.

(g) Peroxidase Isoenzymes

The progressive changes of the anodic and cathodic isoperoxidase banding pattern in floral bud callus cultured on MS medium supplemented with 2.0 mg/l IAA and 3% sucrose, and various phenolic acids are illustrated in Figs. 85a-92a.

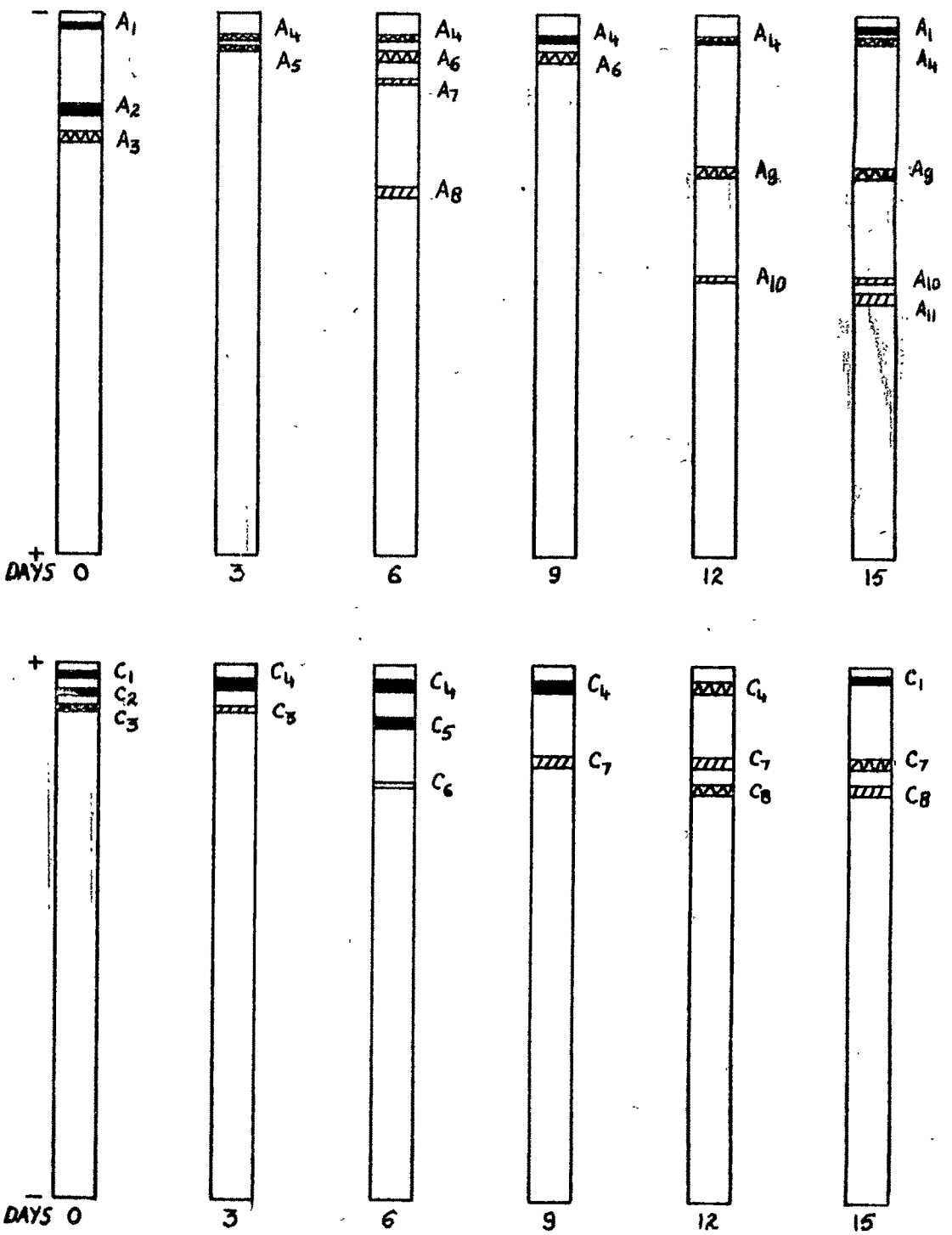
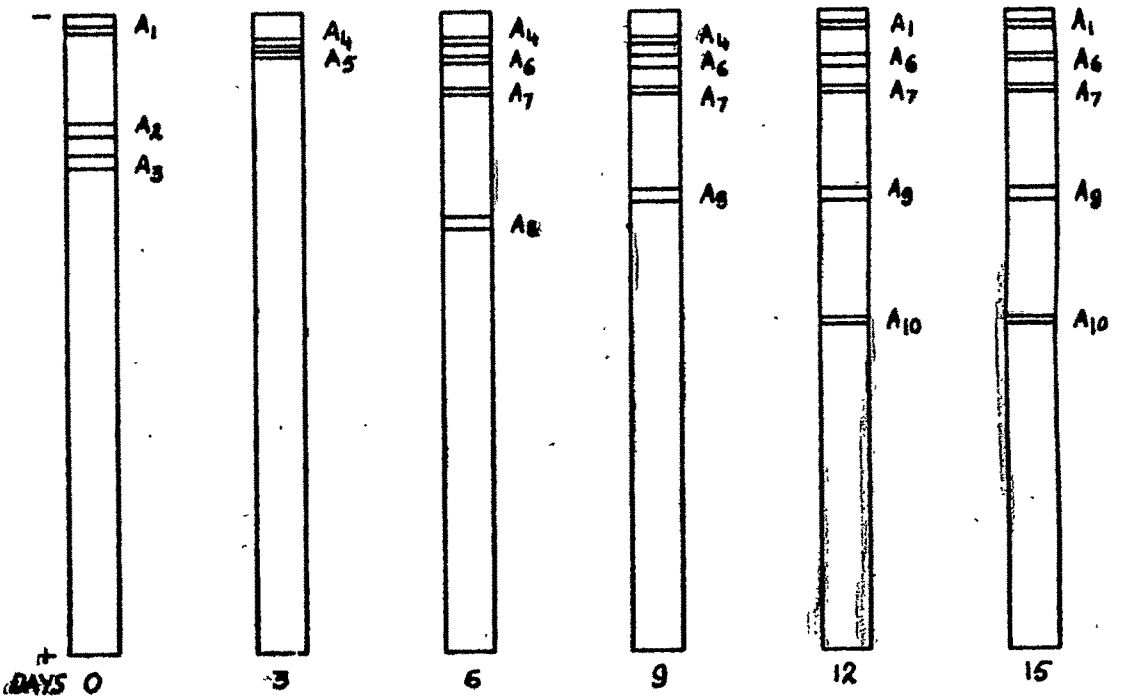


Fig. 85a.

Anodic and Cathodic Isoperoxidase Patterns.
Experimental Details as in Table 23.



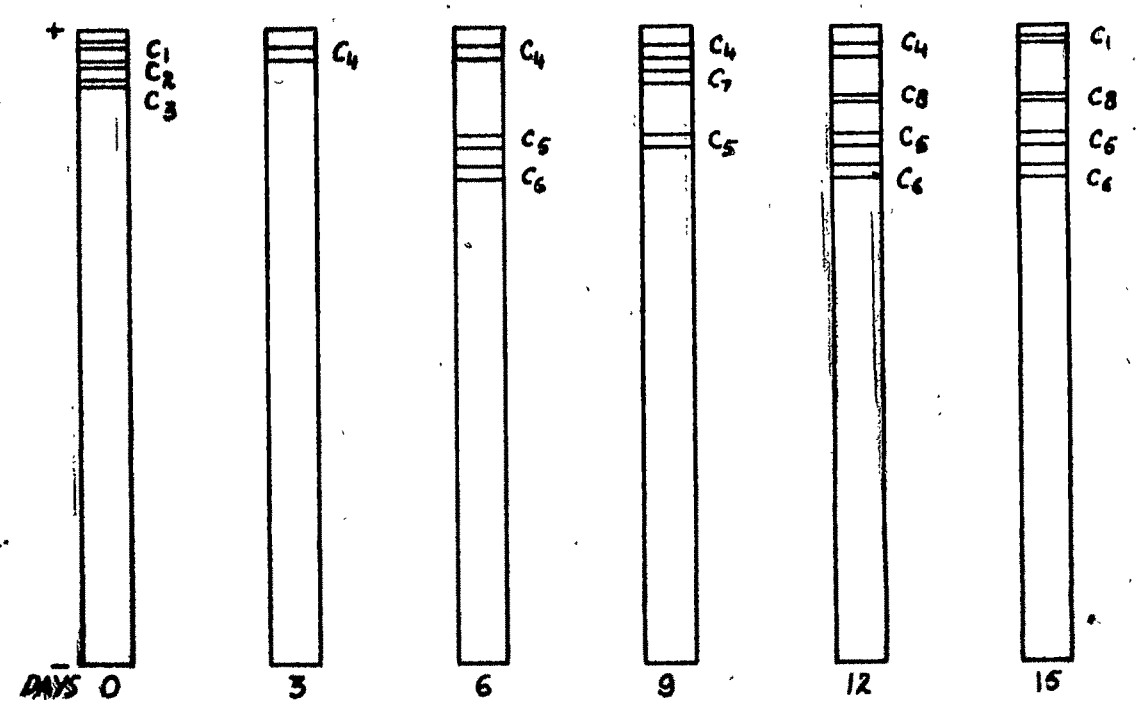


Fig. 86 a.

Anodic and Cathodic Isoperoxidase Patterns.
Experimental Details as in Table 24.

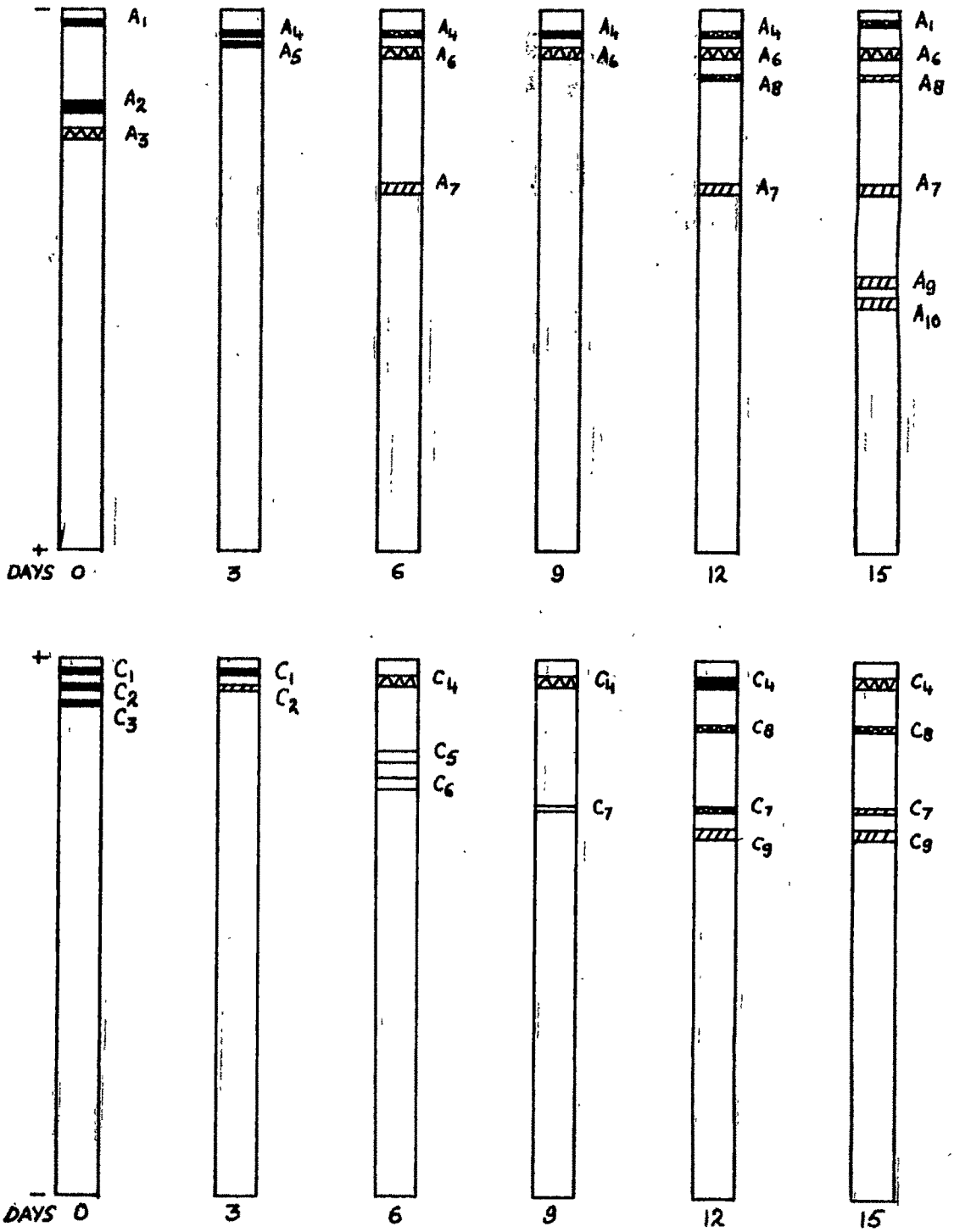
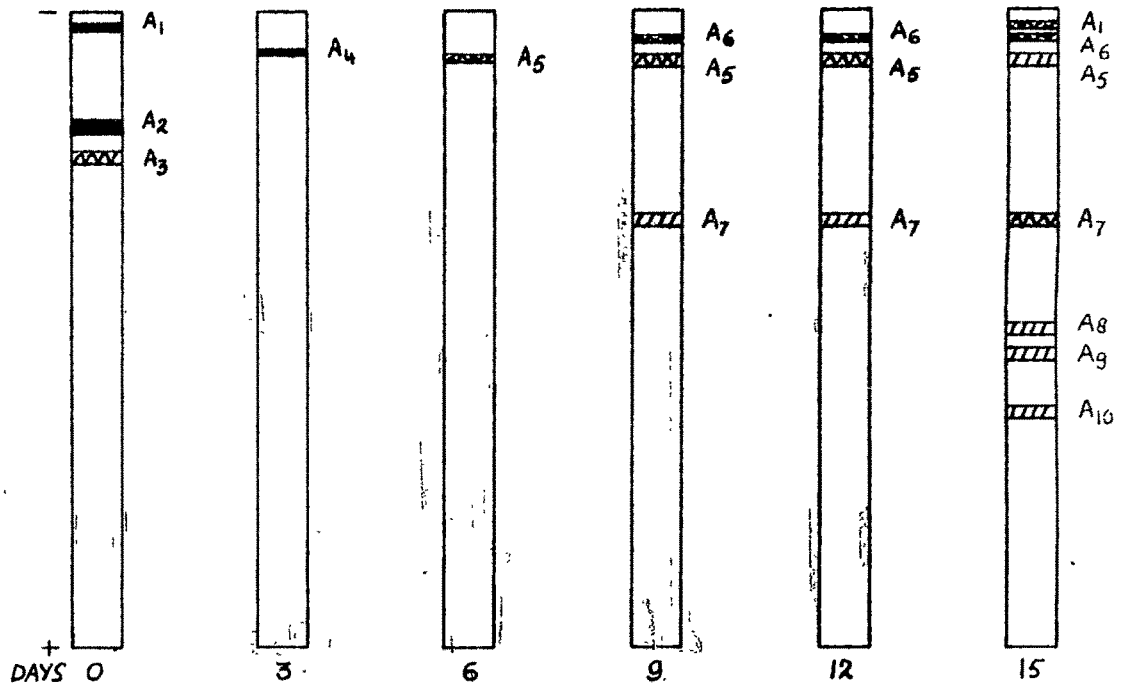


FIG. 87a

Anodic and Cathodic Isoperoxidase Patterns.
Experimental Details as in Table 25.



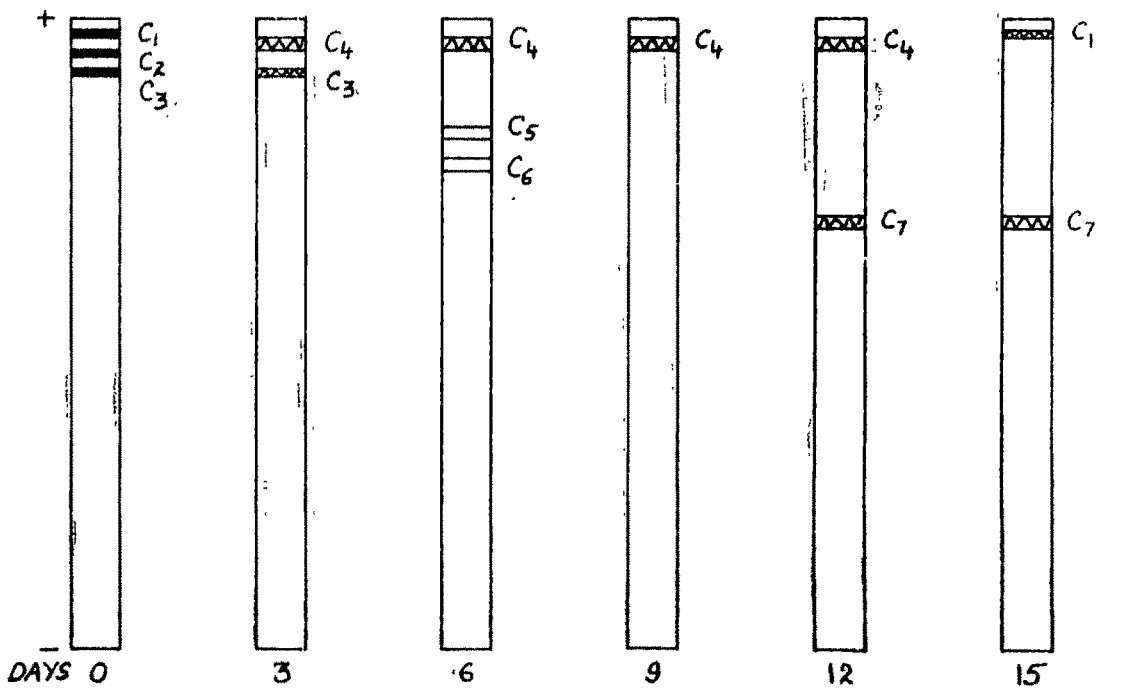
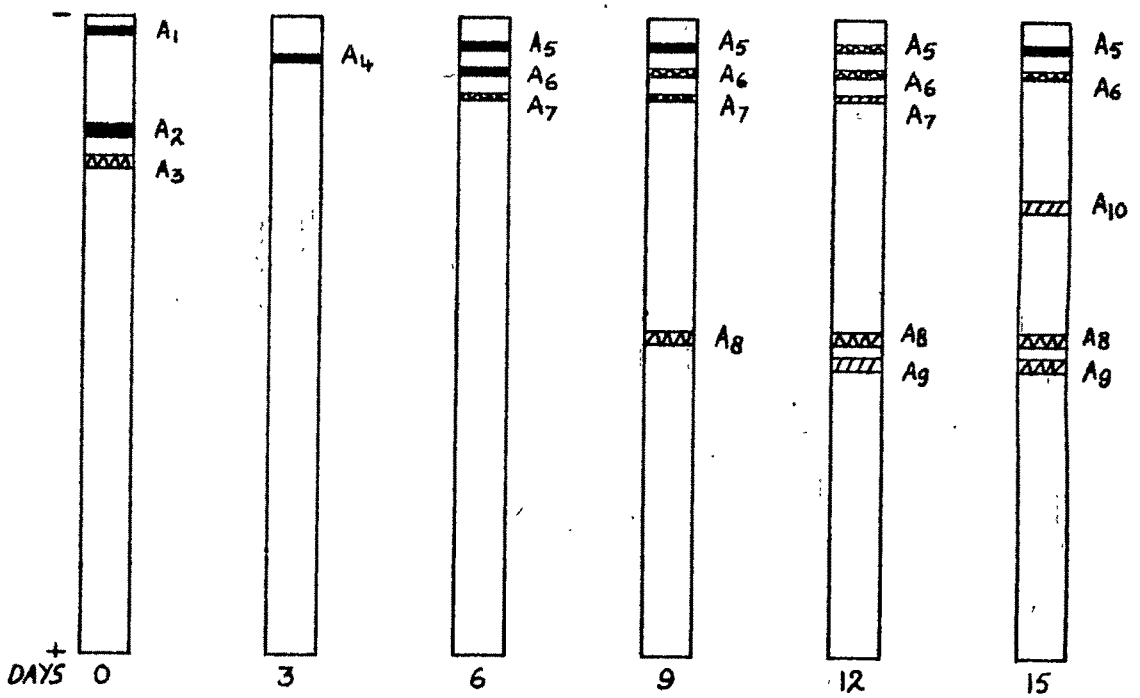


Fig. 88a.

Anodic and Cathodic Isoperoxidase Patterns.
Experimental Details as in Table 26.



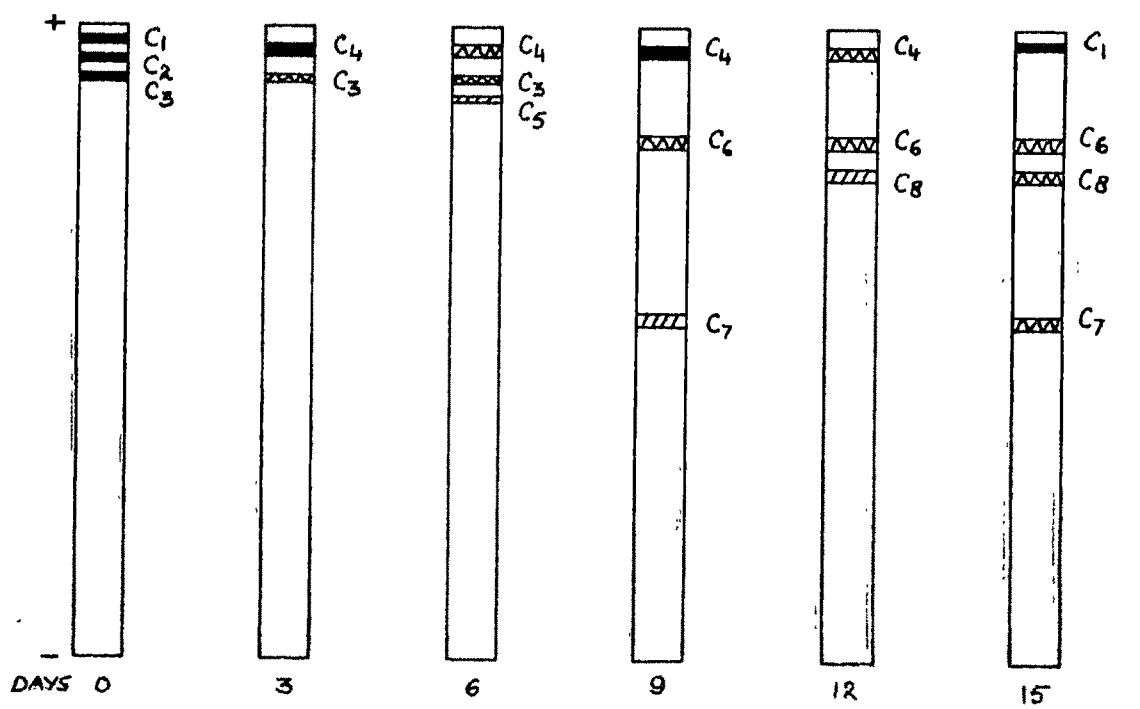


Fig. 89a.

Anodic and Cathodic Isoperoxidase Patterns.
Experimental Details as in Table. 27

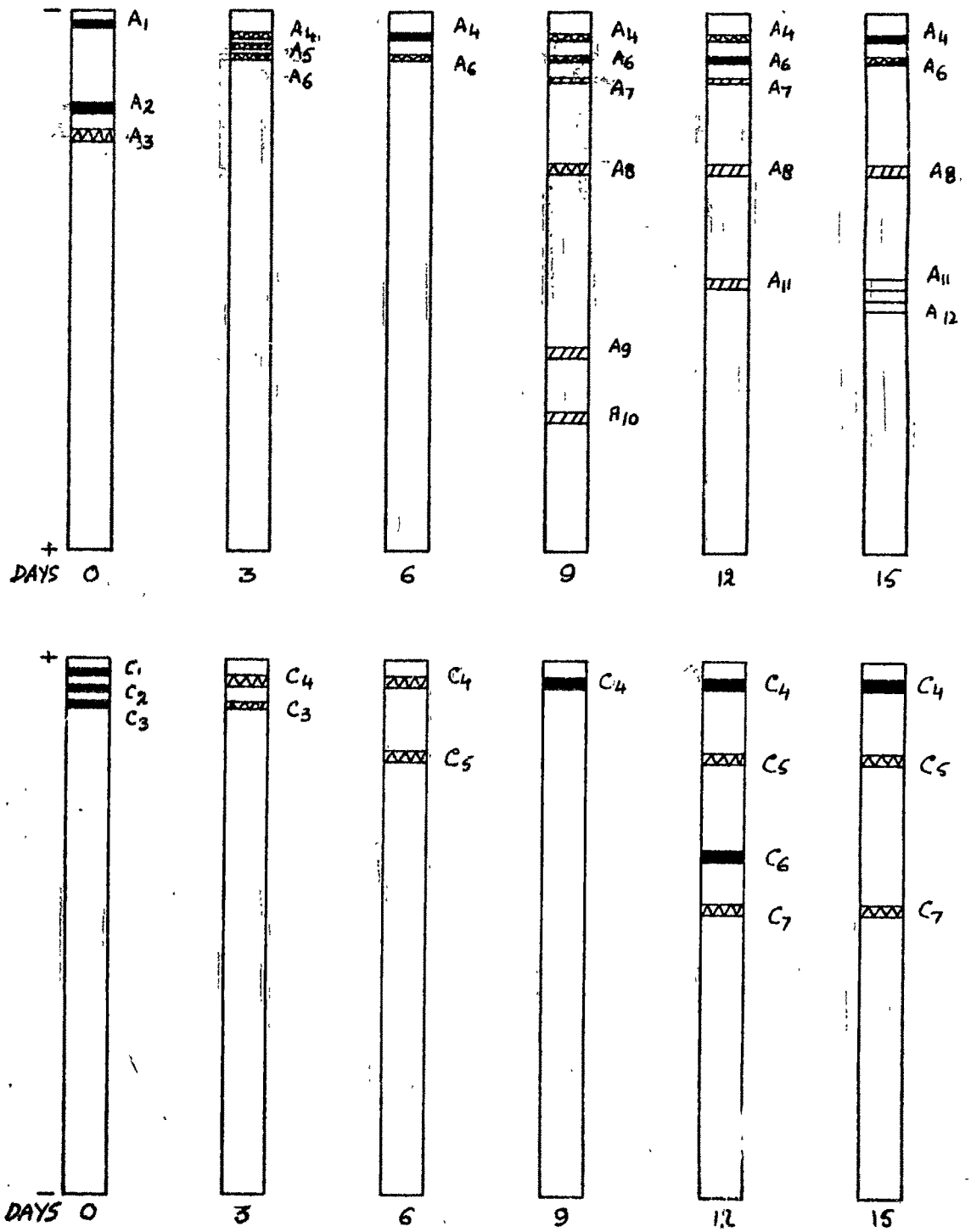
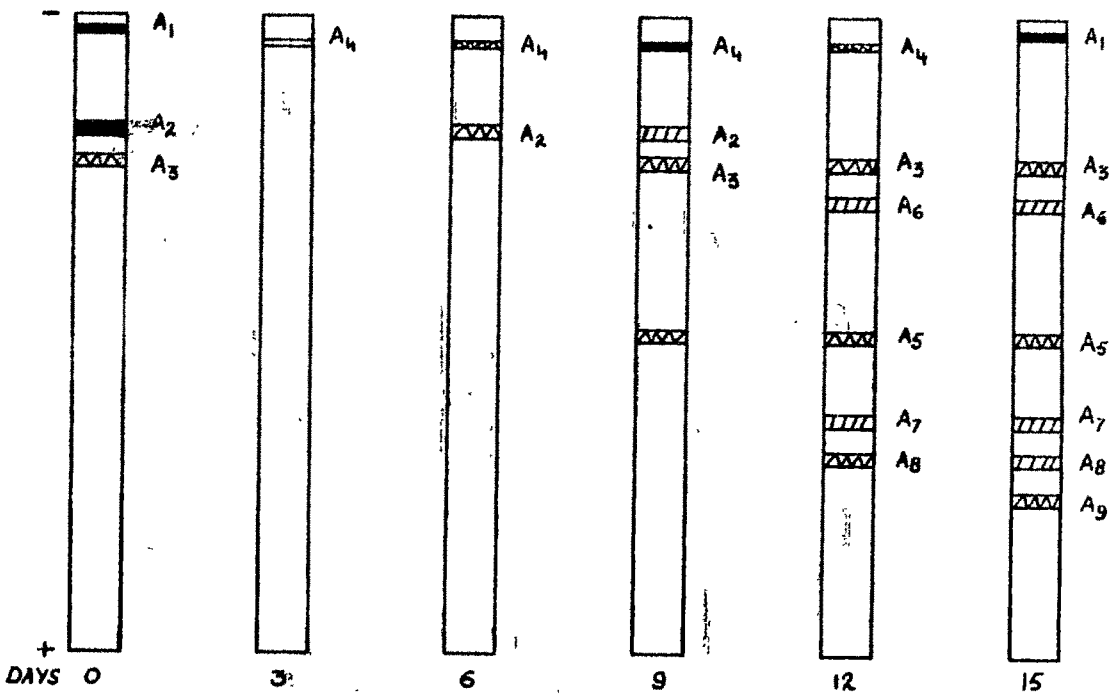
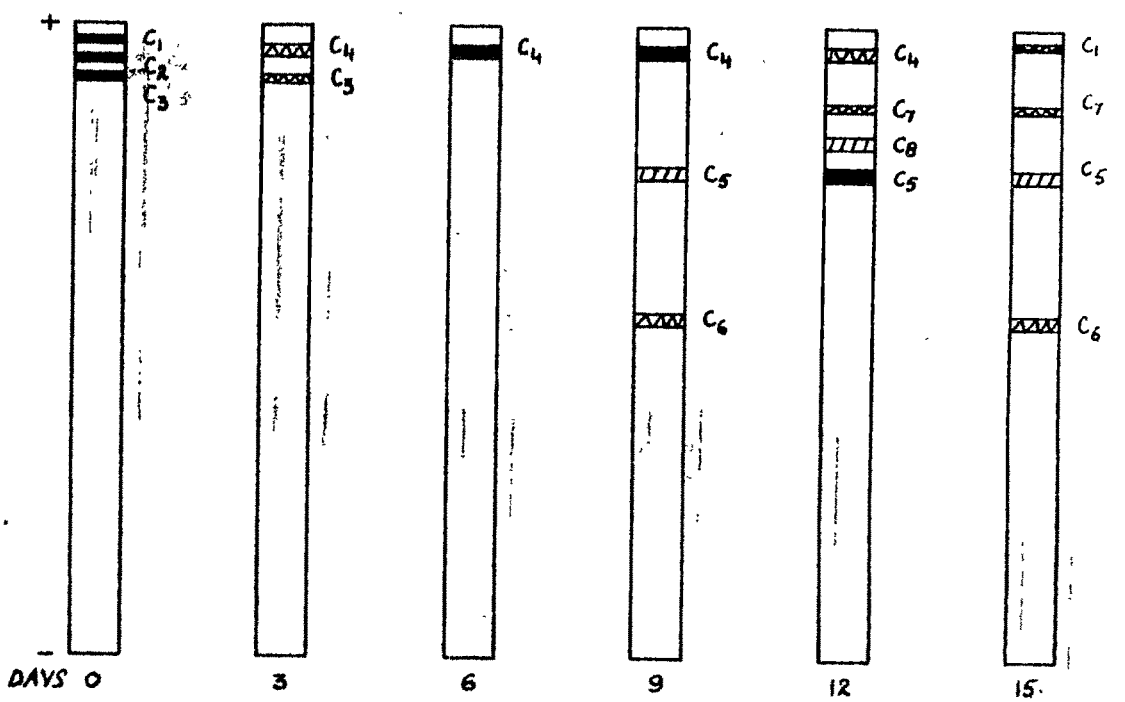


FIG. 90a.

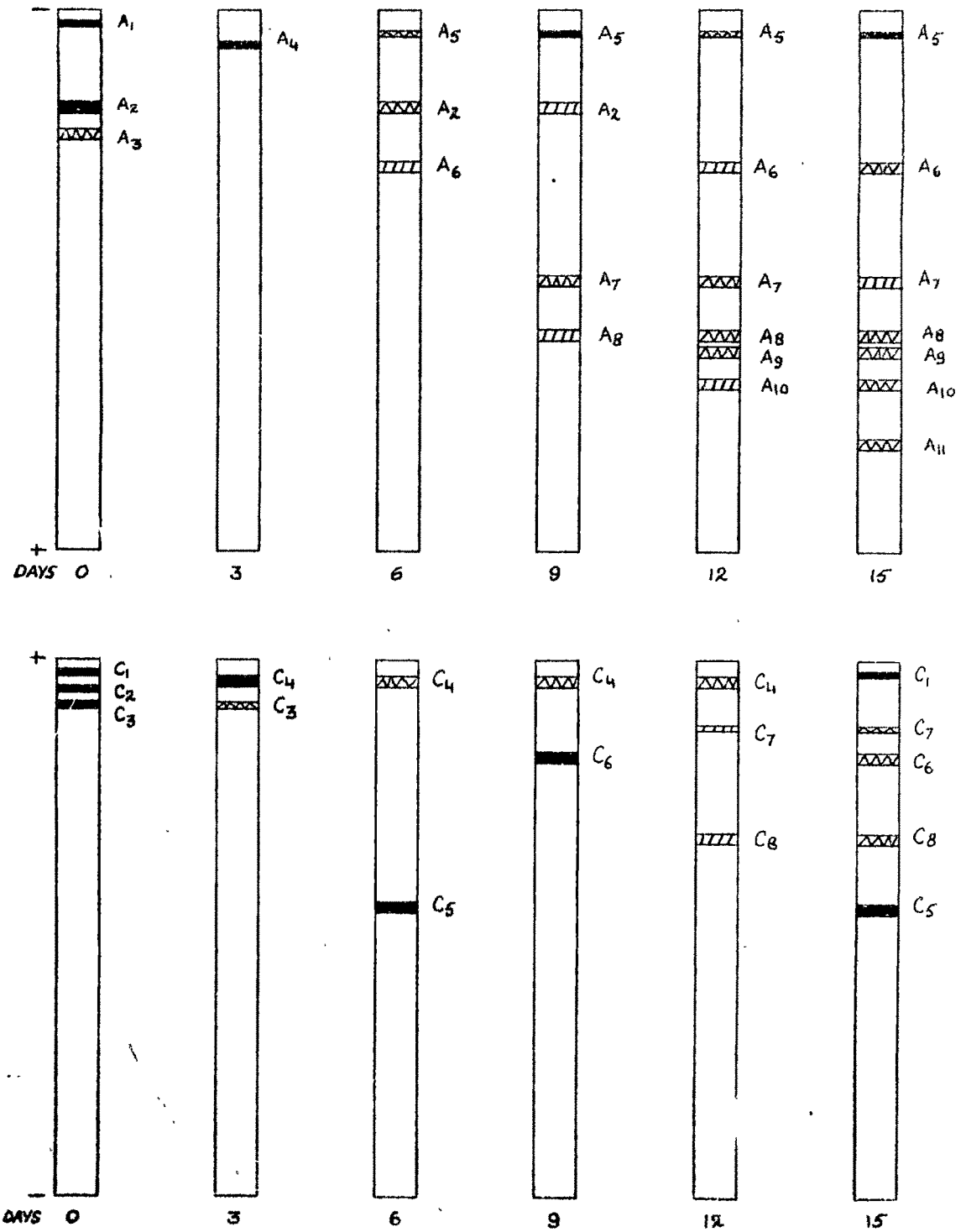
Anodic and Cathodic Isoperoxidase Patterns.
Experimental Details as in Table 28





Anodic and Cathodic Isoperoxidase Patterns, Experimental Details as in Table 29.

FIG. 91a.



Anodic and Cathodic Ikoperoxidase Patterns. Ex'en-mental Details as in Table 30.

FIG. 92 a.

With the addition of 100 μM t-cinnamic acid into the medium (a) the original anodic isoperoxidases A_1-A_3 were repressed till day 12. Only A_1 reappeared on day 15. During the culture period five slow migrating anodic isoperoxidases - A_4 on all days, A_5 on day 3, A_6 on days 6 and 9, A_7 on day 6, and A_8 on day 6 - were synthesized. Two fast migrating isoperoxidases A_{10} on days 12 and 15, and A_{11} on day 15, were also synthesized. The original cathodic isoperoxidases C_1-C_3 were completely repressed till day 12. C_1 reappeared on day 15. Slow migrating cathodic isoperoxidases C_4-C_8 appeared at different cultural periods (Fig. 85a).

As in the previous case the original anodic isoperoxidases A_1-A_3 were completely repressed when the callus was cultured on 500 μM t-cinnamic acid medium (b); A_1, however, reappeared on days 12 and 15. Slow migrating isoperoxidases A_4-A_9 were synthesized at different time intervals. One fast migrating anodic isoperoxidase appeared on days 12 and 15. The original cathodic isoperoxidase C_1 remained repressed on all days except day 15. The other two original cathodic isoperoxidases C_2 and C_3 remained suppressed throughout the culture period. Slow migrating cathodic isoperoxidases C_4-C_8 appeared at different time intervals (Fig. 86a).

Of the original anodic isoperoxidases A_1-A_3 only anodic isoperoxidase A_1 made its appearance on day 15, when the callus

was cultured on 100 µM caffeic acid medium (c). Slow migrating anodic isoperoxidases A_4-A_8 appeared during the culture period. Two fast migrating anodic isoperoxidases A_9 and A_{10} were synthesized on day 15. Of the original cathodic isoperoxidases C_1 and C_2 appeared on day 3. These two isoenzymes were repressed for the rest of culture period. C_3 was completely eliminated throughout the culture period. Slow migrating cathodic isoperoxidases C_4-C_9 appeared during different intervals of the culture period (Fig. 87a).

With culture of the callus on medium (d) supplemented with 500 µM caffeic acid, the original anodic isoperoxidases A_2 and A_3 were completely repressed. The original anodic isoperoxidase A_1, however, appeared on day 15. Besides a number of slow migrating anodic isoenzymes A_4-A_7, three fast migrating anodic isoenzymes A_8-A_{10} also made their appearance. Of the three original cathodic isoperoxidases, C_3 appeared on day 3 and C_1 on day 15. C_2 remained suppressed throughout the culture period. Slow migrating cathodic isoenzymes of peroxidase C_4-C_7 were synthesized at different cultural periods (Fig. 88a).

With the incorporation of 100 µM ferulic acid in the medium (e), all the three original anodic isoperoxidases were repressed. Besides slow migrating isoperoxidases A_4-A_7 and A_{10}, two fast migrating isoperoxidases A_8 and A_9 were also

synthesized. Of the original cathodic isoperoxidases C_2 remained suppressed throughout the culture period. C_3 was seen on days 3 and 6 and C_1 on day 15. Only one fast migrating cathodic isoperoxidase C_7 was synthesized on days 9 and 15. All other cathodic isoperoxidases synthesized C_4-C_6 and C_8, were slow migrating (Fig. 89a).

As in the previous case, when the callus was cultured on medium (f) supplemented with 500 μM ferulic acid, the original anodic isoperoxidases A_1-A_3 were repressed throughout the culture period. Five slow migrating anodic isoperoxidases A_4-A_8 were synthesized at different cultural periods. Also synthesized were fast migrating isoperoxidases A_9-A_{12} between days 9 and 12. Of the original three cathodic isoperoxidases, only C_3 appeared on day 3. C_1 and C_2 did not appear at any time during the course of culture. Besides three slow migrating cathodic isoperoxidases C_4-C_6, one fast migrating cathodic isoperoxidase C_7 was also synthesized. (Fig. 90a).

With the culture of callus on medium supplemented with 1.0 μM p-hydroxybenzoic acid (g), the original anodic isoperoxidase A_1 was repressed throughout the culture period, except on day 15. A_2 appeared on days 6 and 9, and A_3 on days 9, 12, and 15. During the culture period two slow migrating anodic isoenzymes A_4 and A_6 were synthesized on days 3, 6, 9 and 12, and days 12 and 15 respectively. A_5, a fast migrating

anodic isoperoxidase appeared from day 9 onwards. Other fast migrating anodic peroxidases appeared - A_7 and A_8 on days 12 and 15, and A_9 on day 15. Of the original cathodic isoperoxidases only C_3 appeared on day 3. C_1 and C_2 remained repressed for the entire culture period. One fast migrating cathodic isoperoxidase C_6 appeared on days 9 and 15. Slow migrating cathodic isoperoxidases C_4, C_5, C_7 and C_8 appeared at different cultural periods (Fig. 91a).

With the increase of p-hydroxybenzoic acid level to 10 μM in the medium (h) the original anodic isoperoxidases A_1 and A_3, were completely repressed. A_2 appeared on days 6 and 9. Slow migrating anodic isoperoxidases A_4 on day 3, A_5 on days 6, 9, 12 and 15, and A_6 on days 6, 12 and 15, made appearance. There was a spurt in the synthesis of fast migrating anodic isoperoxidases A_7-A_{11} during the later phase of culture i.e. between days 9 and 15. Of the original three cathodic isoperoxidases, C_1 appeared on day 15, C_3 on day 3, while C_2 remained suppressed throughout the culture period. Slow migrating cathodic isoperoxidases C_4, C_6 and C_7 were synthesized at various cultural periods. Only one fast migrating cathodic isoperoxidase C_5 appeared on days 6 and 15 (Fig. 92a).

The anodic isoperoxidase PR which preceded root differentiation in earlier experiments was not synthesized when phenolic acids were added to the root inducing medium.

Expt. 29. Studies with floral bud callus cultured on root inducing medium containing ½ x and 2 x Mn^{++} ion concentration.

Healthy masses of floral bud callus weighing 300±30 mg by fresh weight were cultured on 20 ml of the root inducing medium i.e. MS basal + 2.0 mg/l IAA + 3% sucrose. In the MS basal medium ½ x Mn^{++} and 2 x Mn^{++} ion concentration of the standard level was used. Ordinarily MS basal medium contained 22.3 mg/l of $MnSO_4$. Consequently to attain ½ x and 2 x Mn^{++} level in the medium, respectively 11.15 mg/l and 44.6 mg/l $MnSO_4$ was added in the basal medium.

The culture vessels were incubated at 26±2° in continuous light for a period of 15 days. Every three days, 5 replicates were harvested and analysed for growth, peroxidase, IAA oxidase, MDH, PAL, phenolic accumulation and peroxidase isoenzyme patterns. The results are presented in Figs. 93, 94 and Tables 31, 32.

(a) Growth :

Growth of floral bud callus cultured on the root inducing medium (MS basal + 2 mg/l IAA + 3% sucrose) in which ½ x and 2 x Mn^{++} ion concentrations were used, is illustrated in Figs. 93, 94 and Tables 31, 32.

The growth of floral bud callus, both on fresh and dry

weight basis, was inhibited when Mn^{++} ion concentration was either reduced to $\frac{1}{2}$ x or raised to 2 x of its normal level in the MS medium. The fresh weight increased 1.62 and 1.54 fold respectively on these media. The dry weight increases were 3.43 and 3.24 fold respectively on the above mentioned media (Figs. 93, 94; Tables 31, 32). The medium containing normal levels of Mn^{++} ions gave 1.85 fold and 5.11 fold increase in fresh and dry weight respectively. With alteration in the optimum Mn^{++} level the differentiation of roots was completely inhibited.

(b) Peroxidase :

The progressive changes of peroxidase activity in the floral bud callus tissues cultured on the rooting medium - MS basal + 2.0 mg/l IAA + 3% sucrose - in which Mn^{++} ion level was $\frac{1}{2}$ x and 2 x of the optimal Mn^{++} ion level in the MS medium, are illustrated in Figs. 93, 94 and Tables 31, 32.

The total activity of peroxidase, as well as its specific activity in floral bud callus cultured on rooting medium with $\frac{1}{2}$ x Mn^{++} ion concentration, declined by day 3. The activity on both accounts reached its first peak value on day 6. Between days 6 and 9 the enzyme activity decayed again, rather rapidly. Subsequent to sharp increase in the peroxidase activity, total and specific, the second peak value was attained on day 15 (Fig. 93, Table 31).

Table : 31. Growth, Polyphenols and progressive changes in the Activity of Peroxidase, IAA Oxidase, MDH and PAL in Diploid callus tissues of N. tabacum L. var. Anand-2 cultured on root differentiating medium containing sub-optimum level of Mn^{++} ion.

Medium : MS (containing $\frac{1}{2}$ x Mn^{++} ion conc.) + 2.0 mg/l IAA + 3% sucrose

Incubation : 15 days at 26±2° in continuous light

Inoculum : 300±30 mg fresh tissue

Day	Fresh weight mg/cult.	Dry weight mg/cult.	PEROXIDASE		IAA OXIDASE		MDH		PAL		PHENOLICS	
			units/ cult.	units/mg protein	units/ cult.	units/mg protein	units/ cult.	units/mg protein	units/ cult.	units/mg protein	mg/ cult.	mg%
0	300 (±30)	12 (±3)	1.77	0.92	0.57	0.30	1.69	0.88	0.48	0.25	0.22	1.87
3	317.9 (±18.6)	15.98 (±2.8)	0.69	0.62	4.80	4.36	1.18	1.07	2.68	2.42	0.43	2.72
6	370.7 (±30.8)	25.55 (±2.4)	1.95	2.43	2.64	3.31	5.34	6.68	3.44	4.31	1.01	3.98
9	442.4 (±27.8)	35.28 (±4.0)	0.36	0.42	1.77	2.06	0.65	0.76	3.75	4.37	1.41	4.02
12	474.2 (±43.5)	39.26 (±3.2)	0.80	0.98	4.60	5.62	1.01	1.24	1.16	1.42	1.61	4.11
15	487.7 (±38.3)	41.12 (±5.3)	3.15	2.67	7.30	6.19	10.18	8.63	1.15	0.98	1.11	2.72

Figures in parenthesis represent standard error.

Data represented is average of 5 replicates.

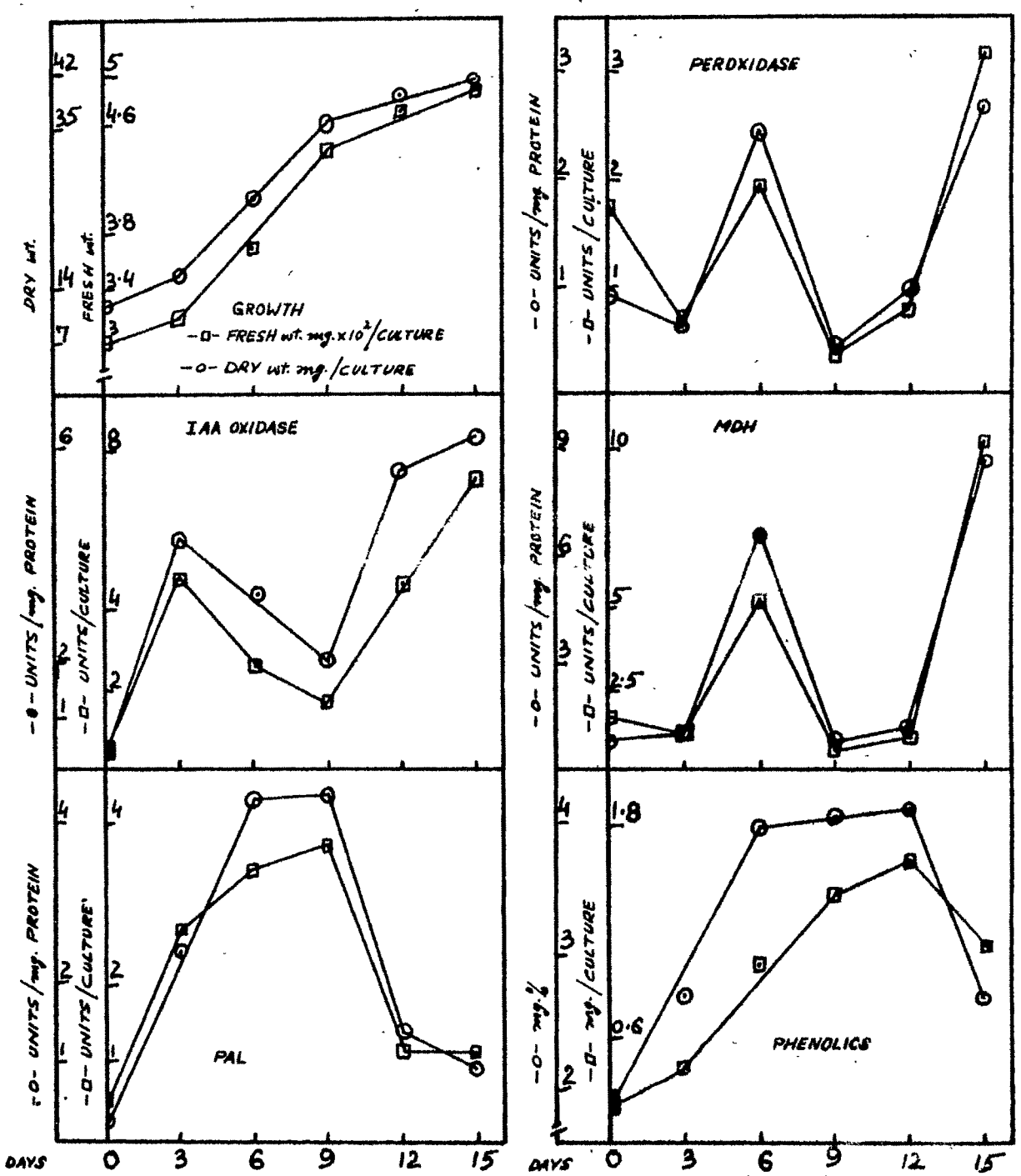


FIG. 93.

Experimental Details as in Table. 31

Table : 32. Growth, Polyphenols and progressive changes in the Activity of Peroxidase, IAA Oxidase, MDH and PAL in diploid callus tissues of N. tabacum L. var.Anand-2 cultured on root differentiating medium containing supra-optimum level of Mn^{++} ion.

Medium : MS (containing 2 x Mn^{++} ion conc.) + 2.0 mg/l IAA + 3% sucrose

Incubation : 15 days at 26±2° in continuous light

Inoculum : 300±30 mg fresh tissue

Day	Fresh weight mg/cult.	Dry weight mg/cult.	PEROXIDASE		IAA OXIDASE		MDH		PAL		PHENOLICS	
			units/ cult.	units/mg protein	units/ cult.	units/mg protein	units/ cult.	units/mg protein	units/ cult.	units/mg protein	mg/ cult.	mg%
0	300 (±30)	12 (±3)	1.77	0.92	0.57	0.30	1.69	0.88	0.48	0.25	0.22	1.87
3	327.8 (±32.0)	20.72 (±1.6)	2.06	5.29	0.92	2.36	1.80	4.62	1.72	4.45	0.59	2.85
6	367.4 (±18.4)	23.99 (±2.8)	1.07	0.86	5.85	4.68	2.56	2.05	4.01	3.21	0.87	3.65
9	404.3 (±30.3)	27.48 (±2.1)	0.81	0.73	7.68	6.92	1.98	1.79	1.36	1.23	0.81	2.98
12	436.7 (±43.2)	30.76 (±3.0)	0.39	1.24	2.31	7.23	0.93	2.92	0.28	0.88	0.71	2.32
15	463.8 (±46.6)	38.92 (±2.7)	1.63	2.21	2.70	3.66	2.85	3.86	1.01	1.37	0.74	1.91

Figures in parenthesis represent standard error.

Data represented is average of 5 replicates.

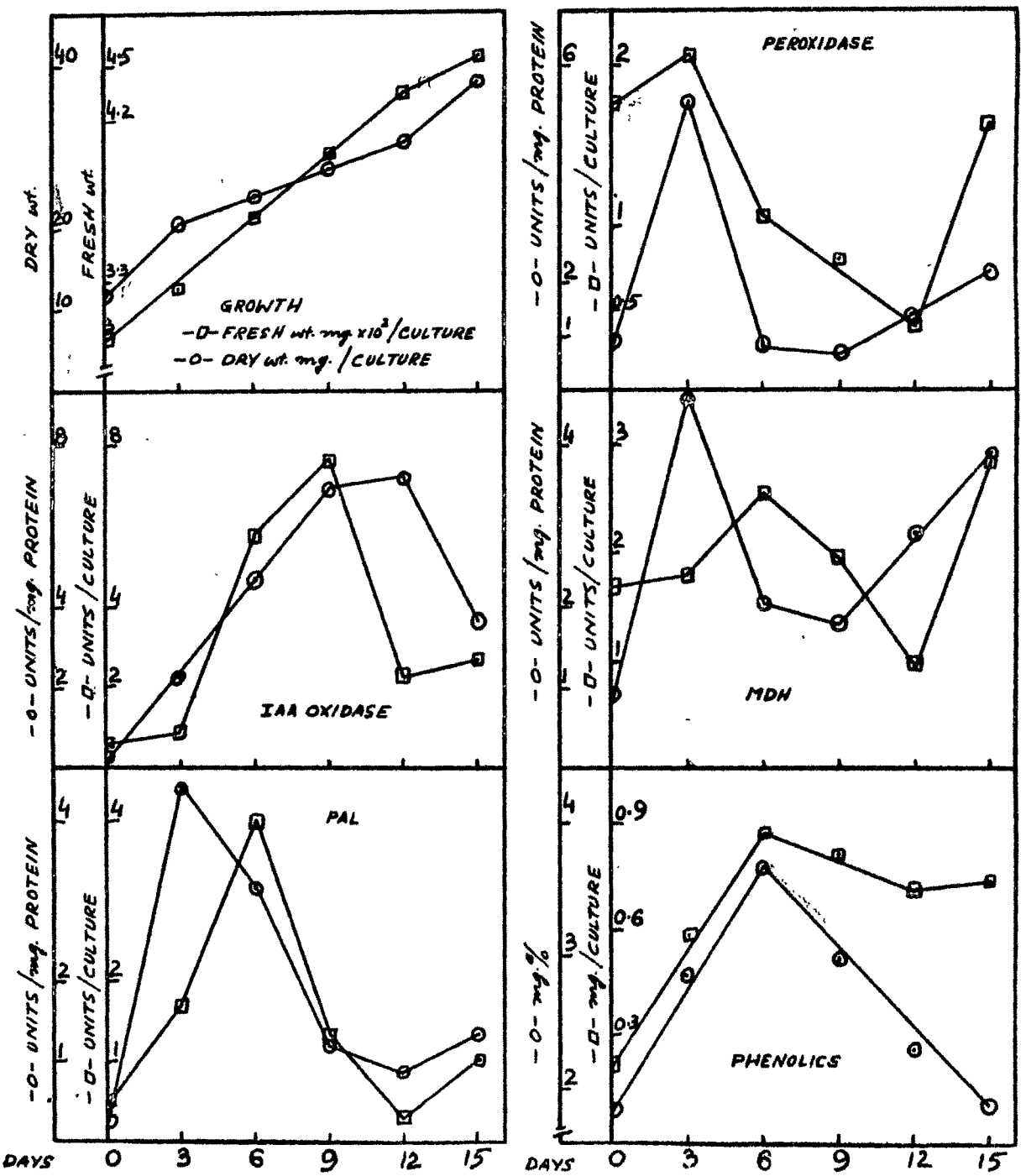


FIG. 94.

Experimental Details as in Table 32.

.The total and specific activity of peroxidase in floral bud callus on 2 x Mn^{++} ion containing medium, also showed double peak pattern. The total peroxidase activity reached the first peak on day 3. The activity, however, decayed rapidly till day 12. Between days 12 and 15 the enzyme activity increased again to reach the second peak. The specific activity of peroxidase also reached its first peak value on day 3. Between days 3 and 6 the enzyme activity decayed sharply. It continued to decline till day 9. Between days 9 and 15 the specific peroxidase activity increased linearly to the second peak value (Fig. 94, Table 32).

The development of peroxidase activity per unit protein between days 12 and 15, on standard root inducing medium and the root inducing medium containing $\frac{1}{2}$ x and 2 x Mn^{++} ion concentrations, was identical in view of the fact that in all the three cases, the enzyme activity was on increase. However, the enzyme activity during this period, when root differentiation occurred on standard root inducing medium, was much higher on media containing $\frac{1}{2}$ x and 2 x Mn^{++} ion level, than on the standard root inducing medium which supported normal Mn^{++} ion level.

(c) IAA Oxidase :

In the floral bud callus cultured on the rooting medium -

MS basal + 2.0 mg/l IAA + 3% sucrose - in which ½ x and 2 x Mn^{++} ion concentrations were used, the IAA Oxidase activity, total and specific, is illustrated in Figs. 93, 94 and Tables 31, 32.

The total and specific activity of IAA Oxidase in floral bud callus cultured on ½ x Mn^{++} ion containing rooting medium showed typical double-peak developmental pattern. The first peak value was reached on day 3 and the second on day 15. The enzyme activity declined between days 3 and 9 (Fig. 93, Table 31). On the 2 x Mn^{++} ion containing rooting medium the total IAA Oxidase activity increased till day 9. The increase was rather slow till day 3 and very sharp between days 3 and 9. The peak value was reached on day 9. The total IAA Oxidase activity decayed rapidly thereof till day 12. It again staged a minor comeback by day 15. The specific activity of IAA Oxidase reached the peak value on day 12. The enzyme activity developed rapidly and linearly between days 0 and 9. Between days 9 and 12 the enzyme activity progressed but slowly. Between days 12 and 15 the specific activity decayed rapidly (Fig. 94, Table 32).

In contrast with total IAA Oxidase activity per unit protein, which declined between days 12 and 15 in floral bud callus cultured on standard root inducing medium, the activity increased during the same period in callus tissues cultured on

the same medium but containing only ½ x Mn^{++} ion level. On the root inducing medium containing 2 x Mn^{++} ion level the activity increased between days 12 and 15, and hence was comparable with the pattern of development of enzyme activity on standard root inducing medium. The activity during this period, however, was much lower on standard root inducing medium. The days 12-15 were characterized by being the period of root differentiation from floral bud callus cultured on root inducing medium.

(d) MDH :

The floral bud callus was cultured on root inducing medium - MS basal + 2.0 mg/l IAA + 3% sucrose - with ½ x and 2 x Mn^{++} ion concentration. The progressive changes in the activity of MDH in such cultures are illustrated in Figs. 93, 94 and Tables 31, 32.

On the ½ x Mn^{++} ion containing rooting medium the total MDH activity declined slightly by day 3. It, however, increased to its first peak value by day 6. Between days 6 and 9 the enzyme activity declined sharply and remained more or less steady till day 12. Between days 12 and 15 the activity increased sharply to reach its second peak value. The specific activity of MDH on the same medium followed identical pattern of development, except for the fact that the enzyme activity did not decay between days 0 and 3 (Fig. 93, Table 31).

On the 2 x Mn^{++} ion containing rooting medium the total MDH activity showed two peaks during its development, one each on days 6 and 15. The specific activity also showed double-peak pattern, but here the peak values were attained on day 3 and 15 (Fig. 94, Table 32).

The total MDH activity per unit protein was on increase between days 12-15, in floral bud callus tissues cultured on standard root inducing medium as well as on the same medium but containing $\frac{1}{2}$ x and 2 x Mn^{++} ion concentrations. However, on the latter two media the activity was appreciably higher than on standard root inducing medium.

(e) PAL :

The progressive changes in the PAL activity in floral bud callus cultured on $\frac{1}{2}$ x and 2 x Mn^{++} ion containing rooting medium, are demonstrated in Figs. 93, 94 and Tables 31, 32.

On the $\frac{1}{2}$ x Mn^{++} ion containing rooting medium the total PAL activity increased sharply till day 3. Thereafter till day 9 the increase in the activity was comparatively slow. The peak value was attained on day 9. A sharp decline in the enzyme activity ensued thereafter till day 12, remaining more or less stable between days 12 and 15. The specific PAL activity on the same medium increased sharply and linearly till day 6. After a slight increase in the activity between

days 6 and 9 the peak value was reached. The enzyme activity declined rapidly thereafter till day 15 (Fig. 93, Table 31).

On 2 x Mn^{++} ion containing rooting medium the total PAL activity increased rapidly till day 6 reaching its peak value. Thereafter the enzyme activity declined along a steep gradient till day 12. The enzyme activity increased again thereof till day 15. The specific PAL activity followed a similar pattern but here the peak value was reached on day 3 instead of day 6 as in the total activity of PAL (Fig. 94, Table 32).

The total PAL activity per unit protein was on decline between days 12-15, in floral bud callus cultured on standard root inducing medium and also on the same medium but containing only $\frac{1}{2}$ x Mn^{++} ion level. During the corresponding period, the activity was on increase on medium containing 2 x Mn^{++} ion level. On the root inducing medium containing $\frac{1}{2}$ x and x 2 x Mn^{++} ion levels, the PAL activity was rather higher than on standard root inducing medium.

(f) Phenolics :

Progressive changes in the accumulation of phenolic compounds in floral bud callus cultured on $\frac{1}{2}$ x and 2 x Mn^{++} ion containing rooting medium are illustrated in Figs. 93, 94 and Tables 31, 32.

On $\frac{1}{2}$ x Mn^{++} ion containing rooting medium the phenolics

accumulated from day 0 onwards reaching peak value on day 12. The phenolic accumulation increased from 0.22 mg/culture to 1.61 mg/culture during this period. On percentage basis also, the peak value of phenolic accumulation was reached on day 12. The accumulation of phenolics increased from 1.87 mg% on day 0 to 4.11 mg% on day 12 (Fig. 93, Table 31).

In the floral bud callus tissues cultured on 2 x Mn^{++} ion containing medium the phenolic accumulation was much less as compared with the previous case. The phenolic accumulation reached its peak value of 0.87 mg/culture on day 6. On percentage basis the peak value of 3.65 mg% was also reached on day 6 (Fig. 94, Table 32).

Though phenolic content on percentage basis was on decline between days 12 and 15 in root inducing medium as well as on the same medium but containing $\frac{1}{2}$ x and 2 x Mn^{++} ion levels, it nevertheless, was much higher on standard root inducing medium than on the media wherein Mn^{++} ion level was manipulated.

(g) Peroxidase Isoenzymes :

The progressive changes in the banding patterns of anodic and cathodic isoperoxidases in floral bud callus cultured on the rooting medium containing $\frac{1}{2}$ x and 2 x Mn^{++} ion levels are illustrated in Figs. 93a, 94a.

On the root inducing medium containing $\frac{1}{2}$ x Mn^{++} ion

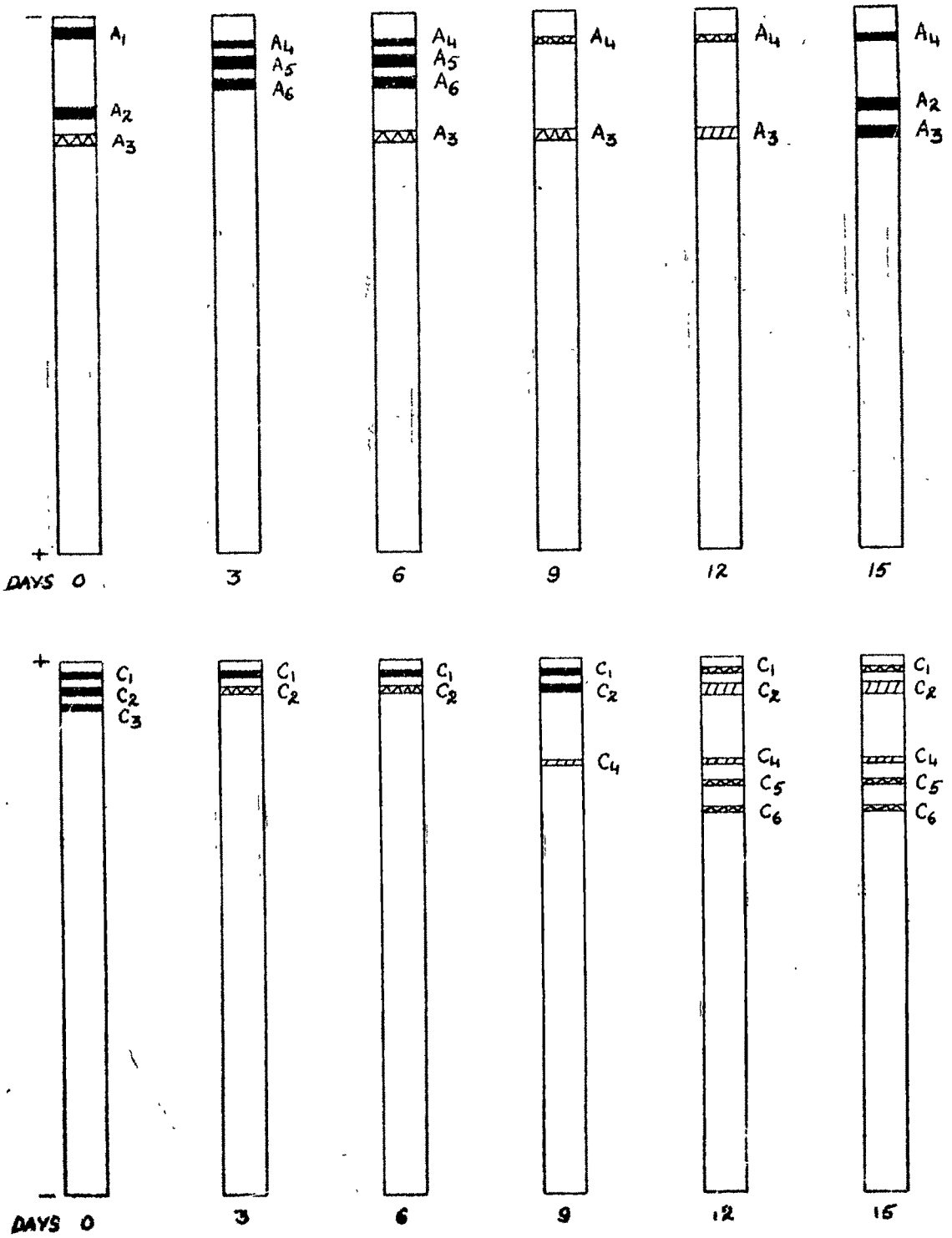


FIG. 93a.

Anodic and Cathodic Isoperoxidase Patterns.

Experimental Details as in Table. 31.

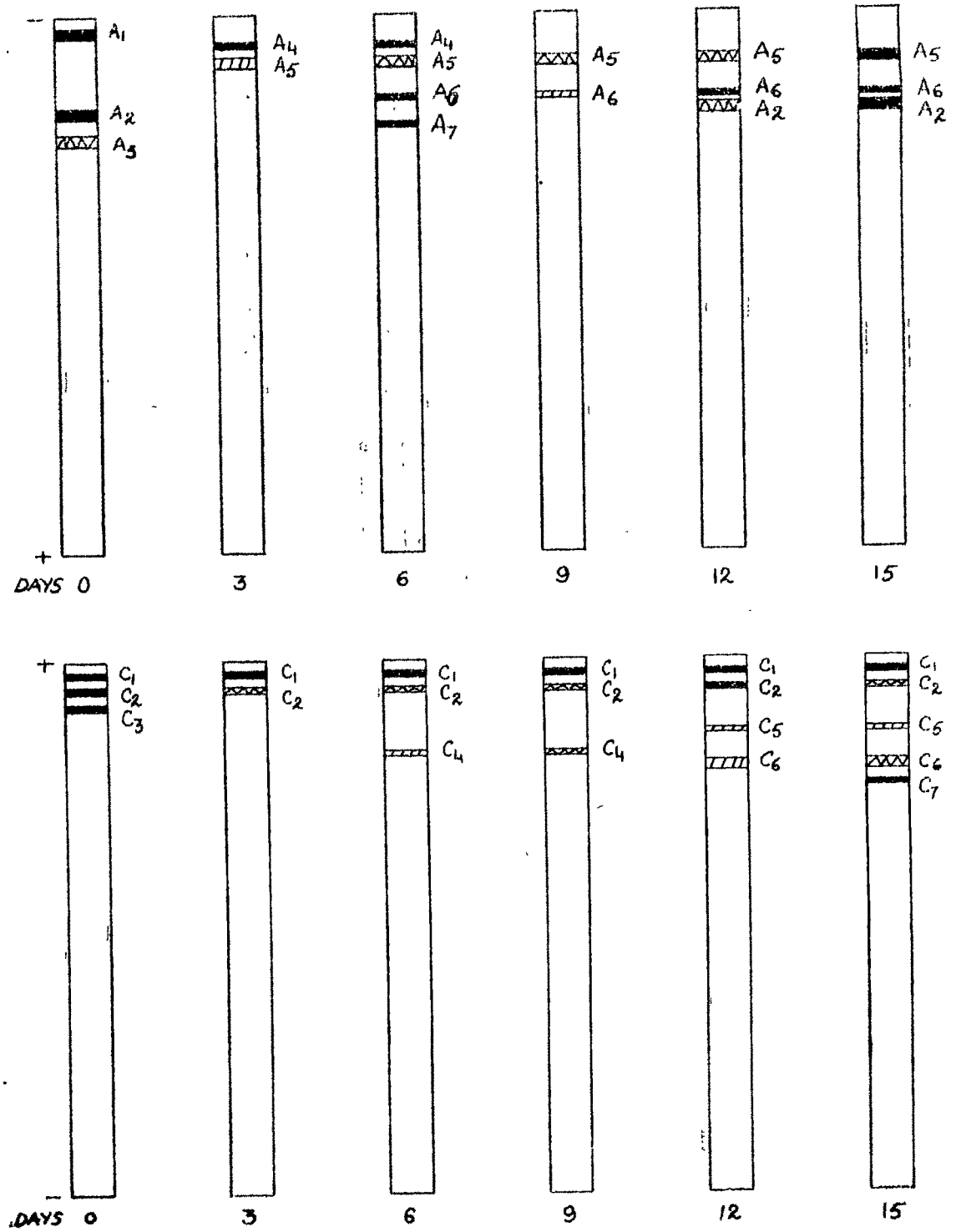


FIG. 94 a.

Anodic and Cathodic Isoperoxidase Patterns

Experimental Details as in Table 32.

the original anodic isoperoxidase A_1 remained suppressed throughout the culture period. The other original anodic isoperoxidase A_2 was synthesized only on day 15; likewise A_3 was synthesized on all days except day 3. During the culture period three slow migrating anodic isoperoxidases, A_4-A_6, were also synthesized. A_4 was synthesized on all days, and A_5 and A_6 on days 3 and 6. The synthesis of the root peroxidase PR was suppressed as was the actual differentiation of roots (Fig. 93a).

On the cathodic scale the original isoperoxidase C_3 remained suppressed right through the culture period. The other two original cathodic isoperoxidases C_1 and C_2 were synthesized on all days of culture. Slow migrating cathodic isoperoxidases C_4-C_6 were synthesized at different times during the culture period. C_4 appeared on days 9, 12 and 15, and C_5 and C_6 on days 12 and 15 (Fig. 93a).

With the use of 2 x Mn^{++} ion in the root inducing medium the original anodic isoperoxidases A_1 and A_3 were suppressed right through the culture period. The third original one A_2 was synthesized on days 12 and 15. Four slow migrating anodic isoperoxidases, A_4-A_7, were synthesized at different time intervals of the culture period. A_4 appeared on days 3 and 6, A_5 on all days of culture, A_6 between days 6 and 15, and A_7 on day 6. As in the previous case the root peroxidase PR, was suppressed as was the differentiation of roots (Fig. 94a).

On the cathodic side the original cathodic isoperoxidase C_3 remained suppressed right through the culture period. The other two original ones C_1 and C_2 were synthesized right through. Four more slow migrating cathodic isoperoxidases, C_4-C_7, were synthesized. C_4 appeared on days 6 and 9, C_5 on days 12 and 15, C_6 on days 12 and 15, and C_7 on day 15 (Fig. 94a).

Expt. 30. Studies with Haploid callus tissues cultured on root inducing medium.

Healthy, green haploid callus masses weighing 300±30 mg were cultured on 20 ml MS basal medium supplemented with 2.0 mg/l IAA and 3% sucrose. The culture vessels were incubated in continuous light at 26±2°. The differentiation of roots from haploid callus tissues occurred any time between days 6 and 9. The experiment was terminated on day 9. Five replicates were harvested every 3 days and analysed for growth, enzymes, isoperoxidase patterns and phenolic content.

(a) Growth :

Growth of haploid tobacco callus cultured on root inducing medium, measured as increase in fresh and dry weights is illustrated in Fig. 95 and Table 33.

Growth, measured on fresh weight basis, was almost linear from day 0 till day 9. Fresh weight increased from 300 mg on day 0 to 852.8 mg on day 9, which was 2.84 fold increase. Dry weight increased between day 0 and day 6 from 12.0 mg to 62.4 mg. An increase of about 5.0 mg in dry weight resulted in subsequent 3 days. Total foldwise increase during the culture period was 5.6.

On this standard root inducing medium the differentiation

of roots occurred any time between days 6 and 9 with around 75% frequency.

The two phases of development,i.e., growth and differentiation are very clearly segregated here. The growth was vigorous during the days preceding root differentiation i.e., between days 0 and 6. However, it slowed down considerably between days 6 and 9, when root differentiation occurred.

(b) Peroxidase :

The development of peroxidase activity in haploid tobacco callus cultured on root inducing medium is illustrated in Fig. 95 and Table 33.

The total peroxidase activity in callus tissues was on increase from day 0 till the end of culture period on day 9. The activity during this period increased about 5.3 folds.

On the other hand, specific peroxidase activity declined between days 0 and 3.The specific activity thereafter increased to its peak value by day 6. Between days 6 and 9 the specific activity, however, declined again.

The peroxidase activity per unit protein was on decrease during the period of root differentiation i.e.,' between days 6 to 9.

Table : 33. Growth, Polyphenols and progressive changes in the Activity of Peroxidase, IAA Oxidase, MDH and PAL during root differentiation from Haploid callus tissues of N. tabacum L. var. Anand-2.

Medium : MS + 2.0 mg/l IAA + 3% sucrose

Incubation : 9 days at 26±2° in continuous light

Inoculum : 300±30 mg fresh tissue

Day	Fresh weight mg/cult.	Dry weight mg/cult.	PEROXIDASE		IAA OXIDASE		MDH		PAL		PHENOLICS	
			units/ cult.	units/mg protein	units/ cult.	units/mg protein	units/ cult.	units/mg protein	units/ cult.	units/mg protein	mg/ cult.	mg%
0	300 (±30)	12 (±3)	0.98	0.86	0.48	0.42	1.05	0.92	0.28	0.25	0.13	1.12
3	470.9 (±24)	29.0 (±4.6)	1.83	0.69	4.88	1.84	2.65	1.0	0.20	0.07	0.27	0.96
6	683.0 (±41.2)	62.4 (±7.1)	3.82	1.26	15.78	5.21	33.31	11.0	3.0	0.99	0.85	1.37
9	852.8 (±38.0)	67.2 (±6.2)	5.20	0.93	6.70	1.21	27.67	5.0	3.40	0.61	1.59	2.38

Figures in parenthesis represent standard error.

Data represented is average of 5 replicates.

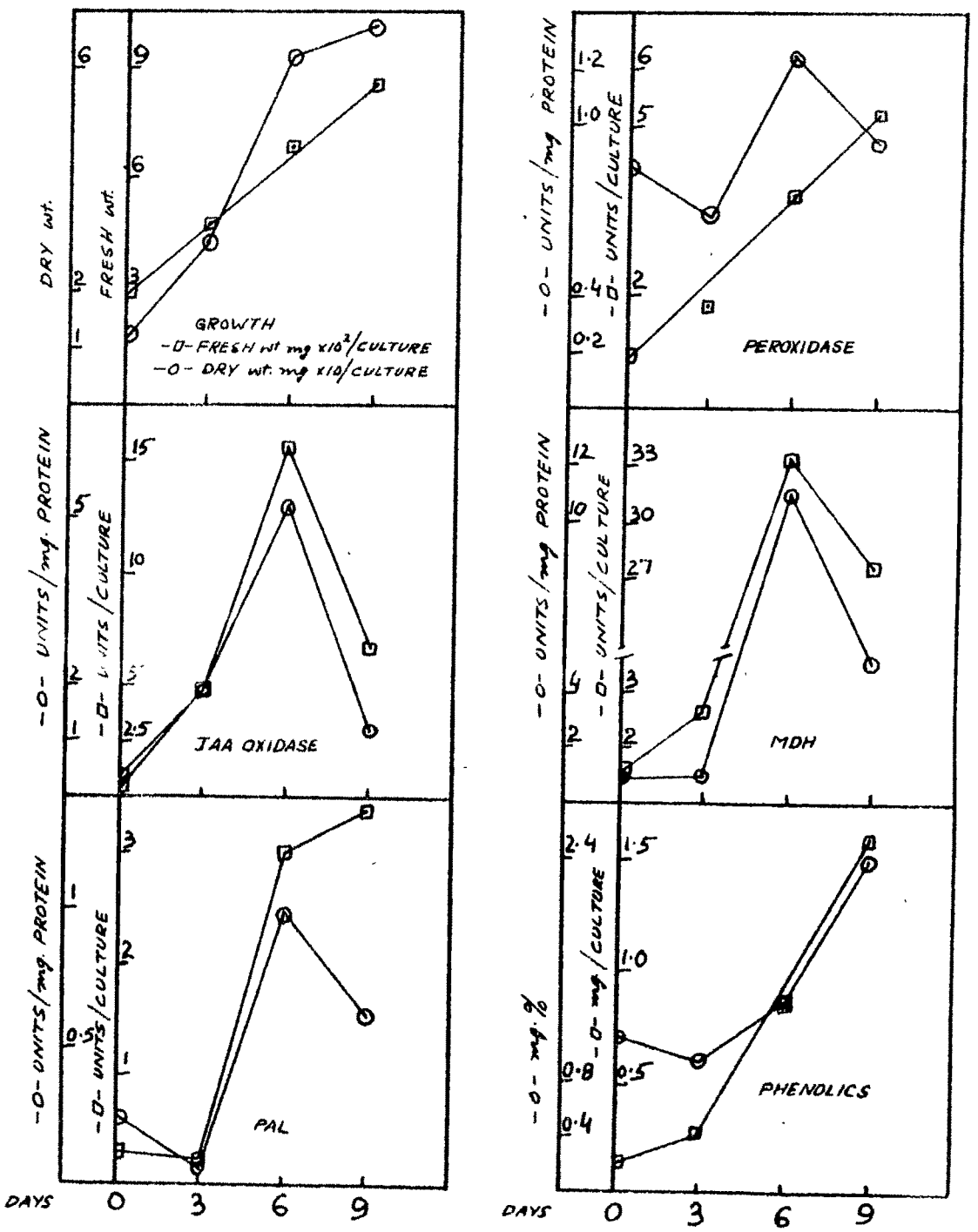


Fig. 95

Experimental Details as in Table 33.

(c) IAA Oxidase :

The progressive changes of IAA Oxidase activity in haploid callus cultured on root inducing medium are illustrated in Fig. 95 and Table 33.

The activity of IAA Oxidase on both accounts i.e., on culture basis and its specific activity, increased to peak values by day 6. Thereafter the activity declined sharply till day 9.

During the period of root differentiation i.e., days 6 to 9, the IAA Oxidase was on the decrease.

(d) MDH :

The progressive changes in total and specific activities of MDH are illustrated in Fig. 95 and Table 33.

The total activity of MDH doubled during the first 3 days of culture. Between days 3 and 6 the enzyme activity shot up to its peak value. During this 3 days period the activity increased by about 12 fold. There after the activity declined till day 9.

The specific activity of MDH in haploid callus increased marginally during the initial 3 days of culture. Between days 3 and 6 the enzyme activity increased sharply (11 fold) to its highest value during the culture period. Thereafter the

specific activity of MDH declined till day 9.

The MDH activity per unit protein and on culture basis registered peak value prior to root differentiation and was on decrease during the period of root differentiation i.e., days 6-9.

(e) PAL :

The progressive changes of total and specific PAL activities in haploid callus cultured on root inducing medium are illustrated in Fig. 95 and Table 33.

The total activity of PAL declined slightly between day 0 and 3. Thereafter the activity increased sharply (nearly 15 fold) till day 6. The activity continued to increase and reached its peak value by day 9. Likewise, the specific activity of PAL declined between day 0 and 3. Thereof, the activity increased till day 6 to reach its peak value before declining again by day 9.

During the root differentiating period i.e., between day 6-9, the PAL activity on culture basis was on increase but the activity per unit protein was on the decline.

(f) Phenolics :

The progressive changes of phenolic accumulation in

haploid callus cultured on root inducing medium are illustrated in Fig. 95 and Table 33.

On culture basis the phenolic content increased continuously between day 0 and day 9, the increase being from 0.13 mg/culture to 1.59 mg/culture. On the contrary, on percentage basis the phenolic content dropped from 1.12 mg% on day 0 to 0.96 mg% on day 3. Thereafter the accumulation of phenolics was on increase till day 9 reaching the peak value of 2.38 mg%.

On per culture basis as well as on percentage basis the phenolic content in haploid callus was on the increase during the period of root differentiation i.e., day 6 to 9.

(g) Peroxidase Isoenzymes :

The progressive changes of anodic and cathodic peroxidase isoenzymes in haploid tobacco tissues cultured on root differentiating medium are illustrated in Fig. 33a.

To begin with on day 0 three anodic isoperoxidases A_1, A_2 and A_3 were present in the haploid callus tissues. Of these A_1 persisted right through the culture period of 9 days. A_2 and A_3 appeared only on day 3 and were suppressed thereafter. On day 3 a slow migrating anodic isoperoxidase A_4 was synthesized. A fast migrating one A_5 also appeared on days 3

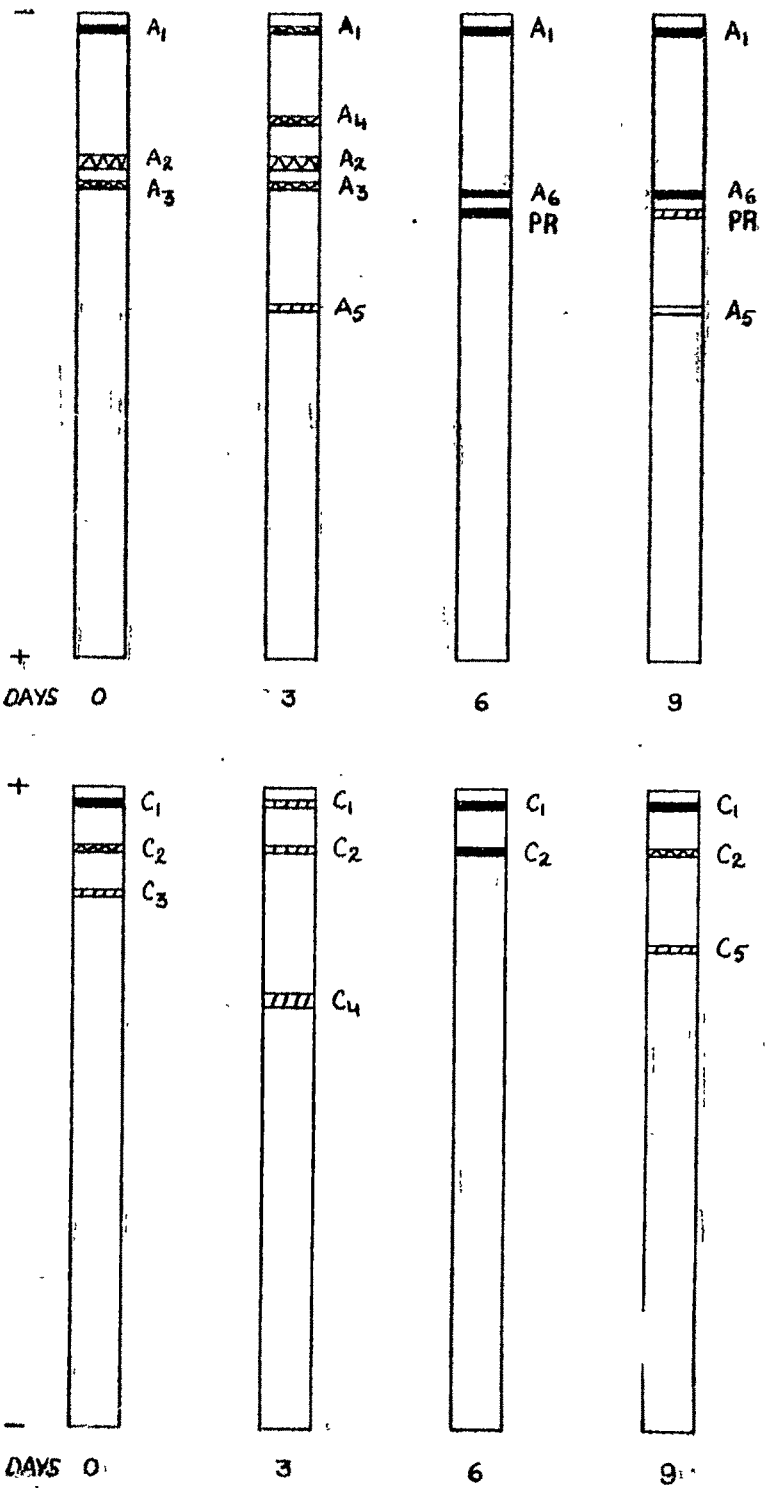


Fig. 95a.

Anodic and Cathodic Isoperoxidase Patterns

Experimental Details as in Table 33..

and 9. Day 6 heralded the synthesis of anodic isoperoxidase A_6, which was a slow migrating one and was synthesized on day 9 also. The isoperoxidase PR was synthesized on day 6 and appeared on day 9 also. This isoperoxidase PR preceded differentiation of roots and was also detected in peroxidase preparation of the differentiated roots.

Initially on day 0 three cathodic isoperoxidases C_1, C_2 and C_3 were present. The cathodic isoperoxidase C_3 was repressed right through the culture period. C_1 and C_2 on the contrary were synthesized on all days of culture. Two slow migrating isoperoxidases, C_4 on day 3 and C_5 on day 9, were also synthesized. Cathodic isoperoxidase pattern did not show any correspondence with root differentiation.

Expt. 31. Studies with haploid callus tissues cultured on root inducing medium supplemented with low and high levels of sucrose.

Healthy and green callus masses of haploid tobacco weighing 300±30 mg by fresh weight were cultured on 20 ml of root inducing medium supplemented with low (1%) and high (6%) sucrose levels. The media used were :

(a) MS basal + 2.0 mg/l IAA + 1% sucrose and

(b) MS basal + 2.0 mg/l IAA + 6% sucrose.

The culture vessels were incubated at 26±2° in continuous light for a maximum period of 15 days. Every 3 days, 5 replicates were harvested and analysed for growth, enzymes, isoperoxidase banding patterns and phenolic accumulation.

(a) Growth :

Growth measured as increase in fresh and dry weights is illustrated in Figs. 96, 97 and Tables 34, 35.

The growth of haploid callus on fresh weight basis was better on 1% sucrose containing medium than on 6% sucrose containing medium. The respective foldwise increasesw were 4.23 and 2.79. The dry mass was more on 6% sucrose medium than on 1% sucrose medium. The corresponding foldwise increases were 6.5 and 5.73

Roots were differentiated from haploid callus tissues on both, 1% and 6% sucrose containing media. The response was, however, delayed as compared with the standard root inducing medium which had the optimum level of sucrose (3%) in it. On 1% sucrose medium roots differentiated on day 15 with 25% frequency, while on 6% sucrose medium on day 12 with 50% frequency.

(b) Peroxidase :

The progressive changes of total and specific activities of peroxidase are illustrated in Figs. 96, 97 and Tables 34,35.

On 1% sucrose containing root inducing medium (MS + 2.0 mg/l IAA), the total peroxidase activity was on the decline between days 0 and 9. From day 9 onwards till day 15 the peroxidase activity increased sharply and linearly to its peak value. The specific activity of peroxidase, however, attained peak value on day 3. Between days 3 and 6 the enzyme activity declined sharply. The decline continued between days 6 and 15 but at a slower rate and along a linear gradient (Fig. 96, Table 34).

The total peroxidase activity on 6% sucrose containing rooting medium demonstrated typical double peaked developmental pattern, one each on days 6 and 15. The specific peroxidase activity, however, reached its peak values on days 3 and 15 (Fig. 97, Table 35).

Table : 34. Growth, Polyphenols and progressive changes in the Activity of Peroxidase, IAA Oxidase, MDH and PAL in Haploid callus tissues of N. tabacum L. var. Anand-2 cultured on root differentiating medium containing low level of sucrose.

Medium : MS + 2.0 mg/l IAA + 1% sucrose

Incubation : 15 days at 26±2° in continuous light

Inoculum : 300±30 mg fresh tissue

Day	Fresh weight mg/cult.	Dry weight mg/cult.	PEROXIDASE		IAA OXIDASE		MDH		PAL		PHENOLICS	
			units/ cult.	units/mg protein	units/ cult.	units/mg protein	units/ cult.	units/mg protein	units/ cult.	units/mg protein	mg/ cult.	mg%
0	300 (±30)	12 (±3)	0.98	0.86	0.48	0.42	1.05	0.92	0.28	0.25	0.13	1.12
3	439.6 (±12.8)	17.2 (±2.6)	0.79	1.10	1.83	2.54	28.76	40.00	3.90	6.03	0.33	1.97
6	522.9 (±18.2)	33.4 (±3.1)	0.73	0.57	0.57	0.44	48.67	37.92	1.16	0.90	0.21	0.65
9	761.9 (±26.8)	41.3 (±4.3)	0.68	0.45	0.83	0.55	54.86	36.00	4.22	2.77	0.46	1.12
12	993.6 (±43.6)	53.8 (±4.1)	1.69	0.35	5.56	1.16	208.80	43.86	16.05	3.37	1.01	1.88
15	1269.9 (±82.9)	68.8 (±4.0)	2.28	0.27	16.16	1.97	442.59	54.00	33.85	4.13	1.52	2.21

Figures in parenthesis represent standard error.
Data represented is average of 5 replicates.

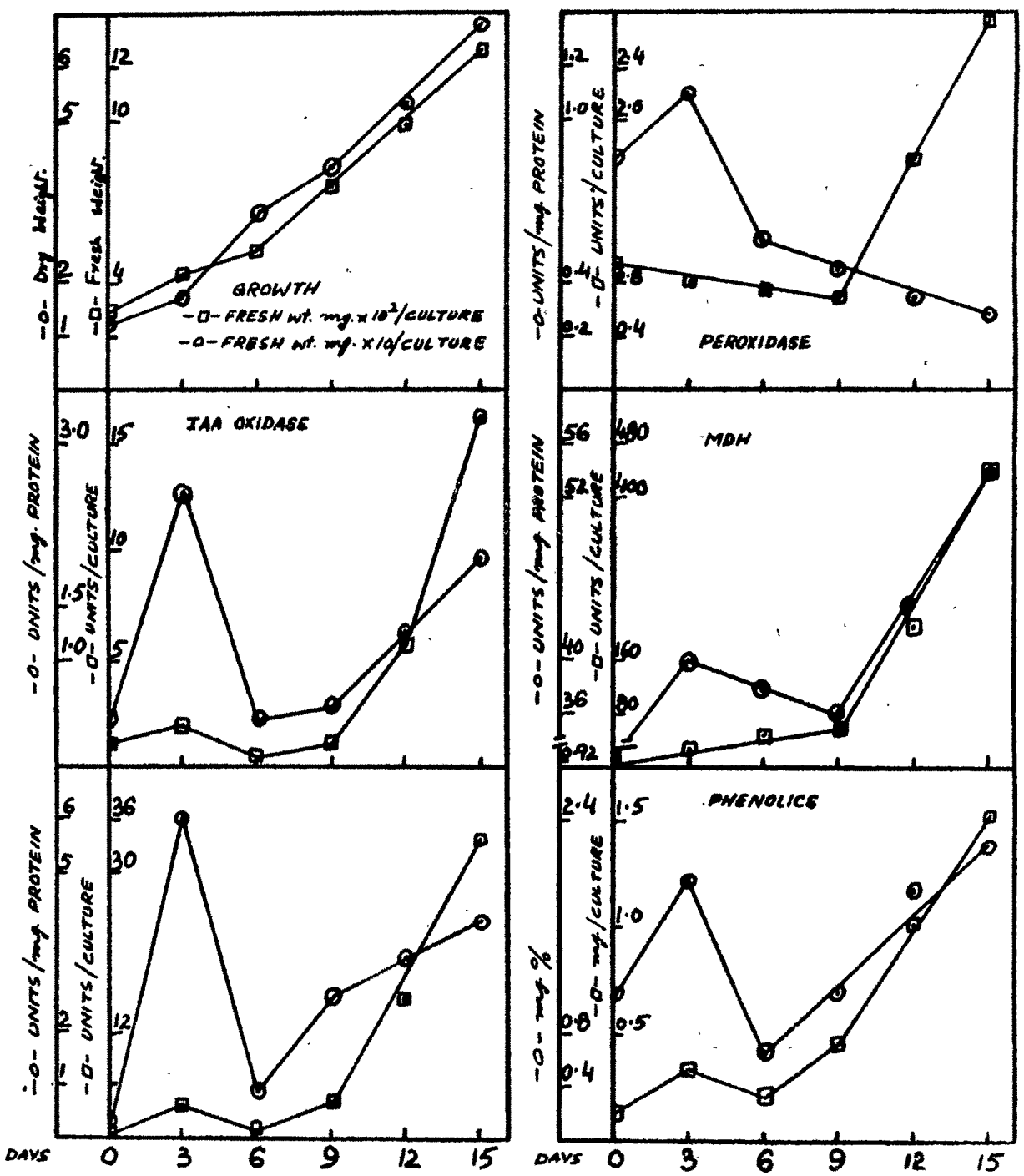


Fig. 96.

Experimental Details as in Table 34.

Table : 35. Growth, Polyphenols and progressive changes in the Activity of Peroxidase, IAA Oxidase, MDH and PAL in Haploid callus tissues of N. tabacum L. var. Anand-2 cultured on root differentiating medium containing high level of sucrose.

Medium : MS + 2.0 mg/l IAA + 6% sucrose

Incubation : 15 days at 26±2° in continuous light

Inoculum : 300±30 mg fresh tissue

Day	Fresh weight mg/cult.	Dry weight mg/cult.	PEROXIDASE		IAA OXIDASE		MDH		PAL		PHENOLICS	
			units/ cult.	units/mg protein	units/ cult.	units/mg protein	units/ cult.	units/mg protein	units/ cult.	units/mg protein	mg/ cult.	mg%
0	300 (±30)	12 (±3)	0.98	0.86	0.48	0.42	1.05	0.92	0.28	0.25	0.13	1.12
3	487.0 (±30.7)	32.0 (±2.2)	1.21	6.87	2.48	14.06	2.53	14.33	18.39	103.92	1.39	4.36
6	545.8 (±27.3)	48.7 (±4.7)	1.74	1.10	5.95	3.74	15.61	9.83	10.91	6.87	1.56	3.21
9	641.0 (±58.1)	59.6 (±4.5)	0.25	0.16	2.67	1.70	7.34	4.66	1.42	0.90	0.63	1.06
12	745.3 (±50.2)	69.3 (±6.8)	2.07	0.38	16.12	2.98	16.53	3.06	6.66	1.23	0.91	1.32
15	838.7 (±62.6)	78.0 (±6.6)	4.02	0.51	15.35	3.81	7.77	1.00	3.81	13.04	1.13	1.46

Figures in parenthesis represent standard error.

Data represented is average of 5 replicates.

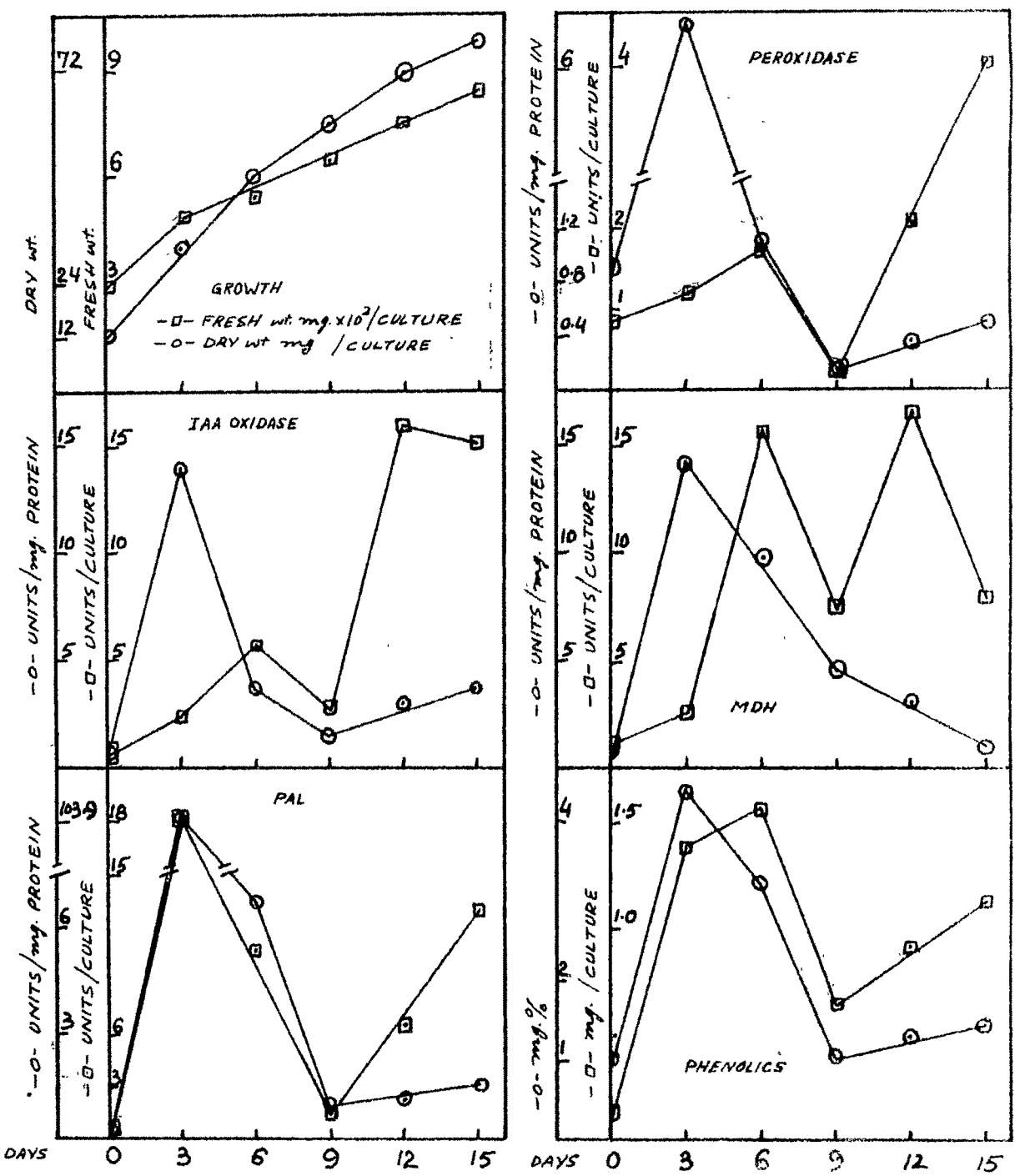


FIG. 97

Experimental Details as in Table 35.

The peroxidase activity per unit protein during root differentiation (day 9-15) on 1% sucrose medium was on the decline whereas on the 6% sucrose medium it was on the increase. Obviously, there was no correspondence between this enzyme and root differentiation.

(c) IAA Oxidase :

The changes in the activity of IAA Oxidase during root differentiation are illustrated in Figs. 96, 97 and Tables 34, 35.

The total and specific activities of IAA Oxidase in haploid tobacco callus on 1% sucrose containing root inducing medium, exhibited double peaked developmental pattern, one each on days 3 and 15 (Fig. 96, Table 34).

On 6% sucrose containing medium also the total and specific activities of IAA Oxidase reached two peak values. The total IAA Oxidase activity reached peak values on days 6 and 12. The specific IAA Oxidase attained peaks on days 3 and 15 (Fig. 97, Table 35).

The IAA Oxidase activity per unit protein during the period of root differentiation (day 9-15) was on the increase on both 1% and 6% sucrose containing root inducing media.

(d) MDH :

The progressive changes of total and specific activities

of MDH in haploid tobacco callus on rooting medium supplemented with low (1%) and high (6%) sucrose levels, are illustrated in Figs. 96, 97 and Tables 34, 35.

The total MDH activity on 1% sucrose containing medium increased along a linear gradient between days 0 and 9. During this period the activity increased from 1.05 to 54.86 units/culture. Between days 9 and 15 the activity showed a very pronounced increase, reaching upto 442.5 units/culture. The specific activity exhibited two peak values on the same medium. Between day 0 and 3 the specific activity increased sharply from 0.92 units/mg protein to 40.0 units/mg protein. Thereafter the activity declined linearly till day 9. Between days 9 and 15 the enzyme activity exhibited another sharp increase, rising from 36.0 units/mg protein to 54.0 units/mg protein (Fig. 96, Table 34).

On 6% sucrose containing medium total MDH activity exhibited double peaked developmental pattern, one each on days 6 and 12. The specific activity demonstrated a single peak on day 3 subsequent to sharp increase of activity. Thereon till day 15 the activity was on the decline (Fig. 97, Table 35). The enzyme activity on 6% sucrose medium was not as high as on 1% sucrose medium.

The MDH activity per unit protein during root inducing

period (day 9-15) was on the increase on 1% sucrose medium and on the decline on 6% sucrose medium.

(e) PAL :

The progressive changes in total and specific activities of PAL in haploid tobacco tissues cultured on root inducing medium supplemented with low (1%) and high (6%) sucrose levels are illustrated in Figs. 96, 97 and Tables 34, 35.

The total and specific activities of PAL in haploid tissues cultured on 1% sucrose medium demonstrated double peaked pattern of development. The first peak was reached on day 3 and the second on day 15 (Fig. 96, Table 34).

On 6% sucrose medium, as in the previous instance, the total and specific activity of PAL demonstrated double peaked pattern of development. The first peak was reached on day 3 as a result of very sharp increase in the activity. Between days 3 and 9 the activity decayed rapidly and considerably. The total activity of PAL increased again between days 9 and 15 along a steep and linear gradient to attain the second peak value by day 15. The specific activity of PAL also increased between days 9 and 15. The increase was linear but marginal (Fig. 97, Table 35).

The PAL activity per unit protein during root differentiating period (day 9-15) was on the increase on both 1% and 6%

sucrose containing media.

(f) Phenolics :

The progressive changes of phenolic accumulation in haploid tobacco callus cultured on root inducing medium containing low (1%) and high 6%) levels of sucrose, are illustrated in Figs. 96, 97 and Tables 34, 35.

On 1% sucrose containing medium the phenolic content increased from 0.13 mg/culture on day 0 to 0.33 mg/culture on day 3. It, however, declined to 0.21 mg/culture by day 6. Between days 6 and 15 the phenolic accumulation was on increase and reached its peak value of 1.52 mg/culture by day 15. On percentage basis the phenolic content increased from 1.12 mg% on day 0 to 1.97 mg% by day 3. The phenolic content, however, declined to 0.65 mg% by day 6. Thereafter, the phenolics continued to accumulate linearly reaching the peak value of 2.21 mg% on day 15 (Fig. 96, Table 34).

On rooting medium containing 6% sucrose the phenolic content in haploid tobacco tissues increased from 0.13 mg/culture on day 0 to 1.56 mg/culture by day 6. Thereafter, the phenolic content declined to 0.63 mg/culture by day 9. Between days 9 and 15 it increased again to 1.13 mg/culture by day 15. On percentage basis the phenolic content shot up from 1.12 mg% on day 0 to 4.36 mg% on day 3. The phenolic content declined

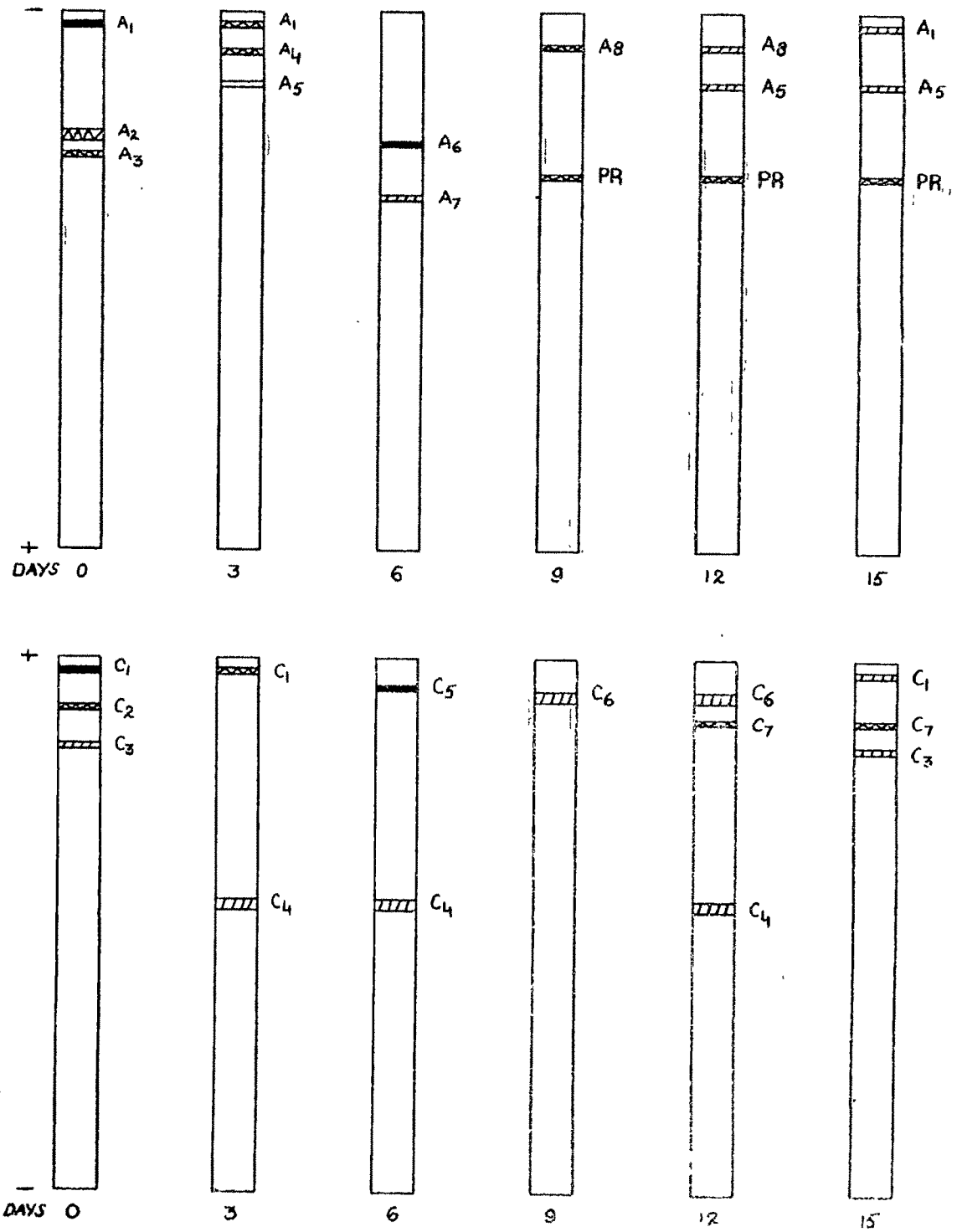
rapidly to 1.06 mg% by day 9. Thereafter, the phenolics accumulated again and reached the value of 1.46 mg% by day 15 (Fig. 97, Table 35).

The phenolic content in haploid tobacco callus tissues during root inducing period (day 9-15) was on increase on both 1% and 6% sucrose containing media.

(g) Peroxidase Isoenzymes :

The progressive changes of the anodic and cathodic isoperoxidases in haploid tobacco callus grown on root inducing medium supplemented with low (1%) and high (6%) levels of sucrose, are illustrated in Figs. 96a, 97a.

On 1% sucrose containing medium of the initial three anodic isoperoxidases A_1, A_2 and A_3, the isoperoxidases A_2 and A_3 were suppressed throughout the culture period. A_1, however, appeared on days 3 and 15. During the culture period five slow migrating anodic isoperoxidases were synthesized. A_4 appeared on day 3; A_5 on days 3, 12 and 15; A_6 and A_7 on day 6; and A_8 on days 9 and 12. Besides, one slow migrating anodic isoperoxidase PR appeared on days 9, 12, and 15. This isoperoxidase was detected in the peroxidase preparations of regenerated roots. On the same medium the original cathodic isoperoxidase C_2 remained suppressed throughout the culture period; C_3 appeared only on day 15 and C_1 on days 3 and 15.



Anodic and Cathodic Isoperoxidase Patterns.

Experimental Details as in Table. 34.

Fig 96a.

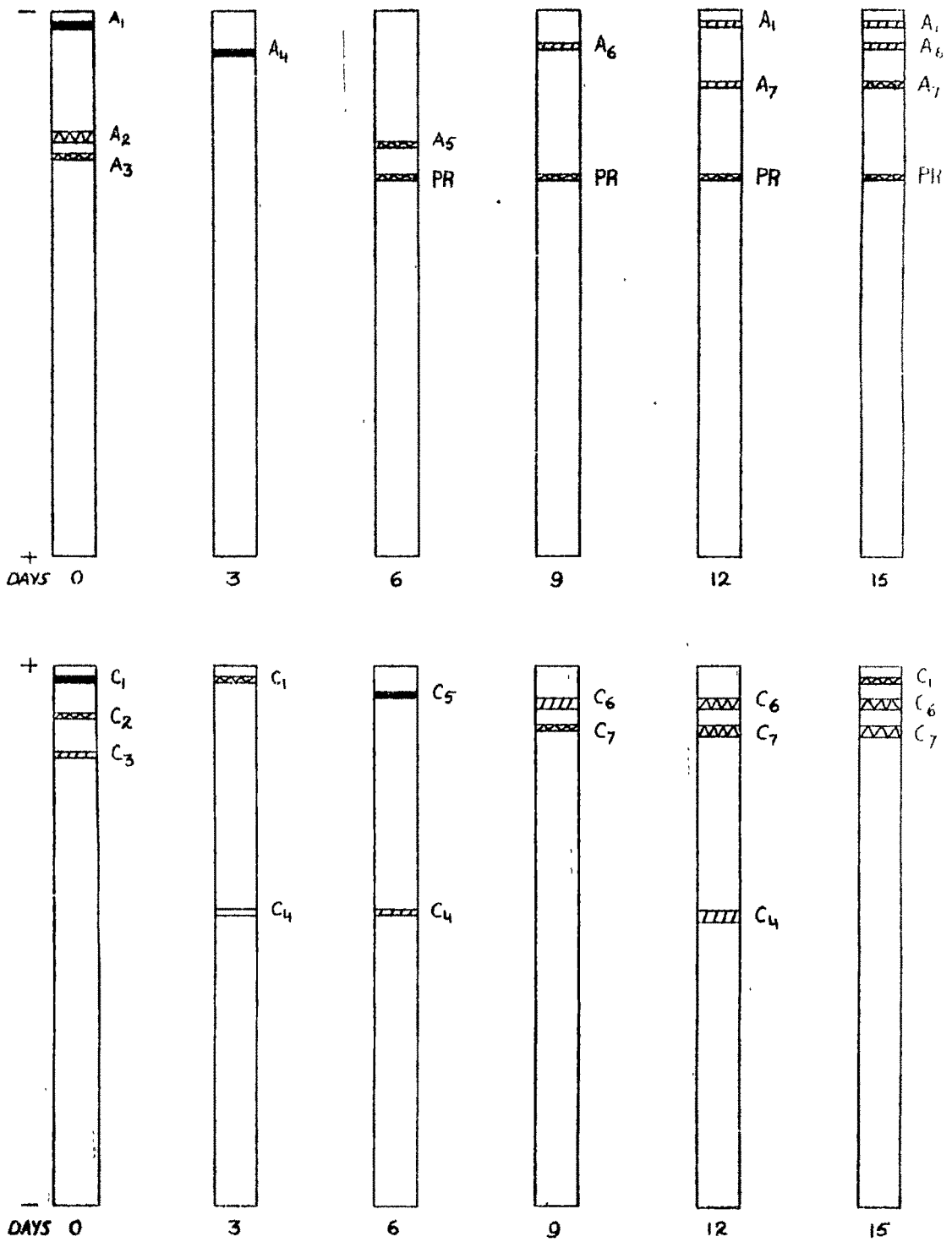


FIG. 97a.

Anodic and Cathodic Isoperoxidase Patterns.
Experimental Details as in Table 35.

During the culture period three slow migrating cathodic isoperoxidases were synthesized. C_5 on day 6, C_6 on days 9 and 12, and C_7 on days 12 and 15. Besides, one fast migrating cathodic isoperoxidase C_4 was synthesized on days 3, 6 and 12 (Fig. 96a).

On 6% sucrose containing medium the original anodic isoperoxidases A_2 and A_3 remained suppressed right through the culture period. The initial anodic isoperoxidase A_1, however, was synthesized on days 12 and 15. Four slow migrating anodic isoperoxidases were synthesized at varying intervals of time during the culture period. A_4 appeared on day 3, A_5 on day 6, A_6 on days 9 and 15, and A_7 on days 12 and 15. Besides, one anodic isoperoxidase PR appeared on days 6, 9, 12 and 15. This isoperoxidase PR was detected in the peroxidase preparations of regenerated roots. Of the initial three cathodic isoperoxidases (C_1, C_2 and C_3), C_2 and C_3 remained suppressed throughout the culture period. The third initial cathodic isoperoxidase C_1 appeared on days 3 and 15. One fast migrating cathodic isoperoxidase C_4 was synthesized on days 3, 6 and 12. Besides, three slow migrating cathodic isoperoxidases C_5, C_6 and C_7 were also synthesized. C_5 appeared on day 6, and C_6 and C_7 on days 9, 12 and 15 (Fig. 97a).

Expt. 32. Studies with haploid callus tissues cultured on root inducing medium supplemented with various phenolic acids.

Healthy callus pieces of haploid tobacco weighing 300±30 mg by fresh weight were cultured on 20 ml of the MS medium containing 2.0 mg/l IAA and 3% sucrose. This medium was further supplemented with phenolic acids in the concentrations enlisted below :

(a) t-cinnamic acid (100 µM)
(b) t-cinnamic acid (500 µM)
(c) caffeic acid (100 µM)
(d) caffeic acid (500 µM)
(e) ferulic acid (100 µM)
(f) ferulic acid (500 µM)
(g) p-hydroxybenzoic acid (1.0 µM)
(h) p-hydroxybenzoic acid (10.0 µM).

The culture vessels were incubated at 26±2° in continuous light. Every 3 days, till day 9, 5 replicates were harvested and analysed for growth, enzymes, peroxidase isoenzymes and phenolic accumulation.

(a) Growth :

Growth, expressed as increase in fresh and dry weights, of haploid tobacco callus cultured on the root inducing

medium (MS basal + 2.0 mg/l IAA + 3% sucrose) in which phenolic acids were added, is illustrated in Figs. 98 - 105 and Tables 36 - 43.

Of all the phenolic acids used, only caffeic acid (500/uM) and p-hydroxybenzoic acid (1.0, 10.0/uM) enhanced growth. Maximum increase in dry weight (6.57 fold) was registered on 1.0/uM p-hydroxybenzoic acid medium. Maximum increase in fresh weight (3.36 fold) occurred on 10.0/uM p-hydroxybenzoic acid medium.

Addition of t-cinnamic acid into the medium at 100 and 500/uM brought about 2.2 and 2.71 fold increase respectively in fresh weight. The corresponding dry weight increases were 4.0 and 5.85 fold respectively (Figs. 98, 99; Tables 36, 37). Root differentiation occurred on day 6 with 75% frequency on root inducing medium containing 100/uM t-cinnamic acid. Incorporation of 500/uM t-cinnamic acid inhibited this response.

Caffeic acid at 100 and 500/uM in the medium increased fresh weight by 2.69 and 3.0 fold, whereas the dry weight increases were 4.83 and 6.3 fold respectively (Figs. 100,101; Tables 38, 39). Root differentiation occurred on day 6 with 50% frequency on incorporation of 500/uM caffeic acid into rooting medium. In 100/uM caffeic acid the morphogenetic response was absent.

On 100 and 500 µM ferulic acid containing root inducing medium fresh weight increased 2.42 and 2.66 fold respectively. The dry mass increases on the two media were 4.3 and 4.58 fold respectively (Figs. 102, 103; Tables 40,41). Addition of ferulic acid (100, 500 µM) into the root inducing medium brought about total inhibition of root differentiation from haploid tobacco callus.

Addition of p-hydroxybenzoic acid (1.0 and 10.0 µM) into the rooting medium resulted in 3.32 and 3.36 fold increases in fresh weight respectively. The corresponding increases in dry weight were 6.57 and 5.71 fold (Figs. 104, 105; Tables 42, 43). Addition of p-hydroxybenzoic acid at both the levels into the rooting medium inhibited root differentiation.

Of significance was the observation that the differentiation of roots was inhibited by the addition of all but two of the phenolic acids. Root differentiation in haploid callus occurred only in the presence of 100 µM t-cinnamic acid and 500 µM caffeic acid in the root inducing medium. In contrast, the differentiation of roots in diploid callus was not registered in presence of any of these phenolic acids.

(b) Peroxidase :

The progressive changes of peroxidase activity in haploid tobacco tissues cultured on root inducing medium supplemented

with different phenolic acids are illustrated in Figs. 98 - 105 and Tables 36 - 43.

With the incorporation of 100 µM t-cinnamic acid into the medium, the total and specific activities of peroxidase declined sharply between days 0 and 6. Thereafter between days 6-9 the peroxidase activity on both accounts increased. The increase of total peroxidase activity was quite substantial whereas the increase in specific activity during the same period was only marginal (Fig. 98, Table 36). The total and specific activities of peroxidase decayed initially with the addition of 500 µM t-cinnamic acid into the medium. The total activity of peroxidase declined slightly by day 3, but staged a minor increase by day 6. Thereafter it shot up to its peak value by day 9. The decrease of specific activity of peroxidase between days 0 and 3 was quite sharp. However, the activity increased thereof. to its peak value by day 9 (Fig. 99, Table 37).

The peroxidase activity per unit protein was on decline before the differentiation of roots (on day 6) on root inducing medium supplemented with 100 µM t-cinnamic acid. On the other hand, the activity was on increase during the same period when 500 µM t-cinnamic acid was added into the root inducing medium. As stated earlier, the later concentration of t-cinnamic acid inhibited root differentiation.

The total peroxidase activity in haploid tobacco callus cultured on root inducing medium supplemented with 100 µM caffeic acid was on increase right from the day 0 of culture. The increase was almost linear till day 9, when it reached its peak value. On the other hand, the specific activity of peroxidase increased rather slowly along a linear gradient till day 6. Thereafter the specific peroxidase activity shot up to its peak value by day 9 (Fig. 100, Table 38).

On 500 µM caffeic acid containing rooting medium the total peroxidase activity remained stable between days 0 and 3, followed by a sharp increase in the activity till day 6. Though the total peroxidase activity continued to increase beyond day 6 till day 9, the increase in the activity was rather modest. The specific activity of peroxidase on the same medium increased to reach its peak value on day 3. Thereafter the activity declined rapidly and linearly till day 9 (Fig. 101, Table 39).

The peroxidase activity per unit protein in haploid tobacco callus during root differentiating period (day 3-9) was on increase with the addition of 100 µM caffeic acid into the medium. During the same period the enzyme activity was on decline when 500 µM caffeic acid was added into the root inducing medium. As mentioned earlier, incorporation of 500 µM caffeic acid into the medium supported root differentiation

whereas the lower level (100 µM) brought about its inhibition.

In haploid callus tissues cultured on 100 µM ferulic acid the total peroxidase activity was on increase from day 0 till day 9. It reached its peak value on day 9. On the other hand, the specific peroxidase activity declined slightly between days 0 and 3. Thereof, it increased sharply to its peak value by day 9 (Fig. 102, Table 40).

Elevation of ferulic acid level to 500 µM in the medium resulted in decay of total and specific activities of peroxidase by day 3. Thereafter, the activities on both accounts demonstrated rapid increase till day 9 (Fig. 103, Table 41).

The peroxidase activity per unit protein in haploid tobacco tissues cultured on medium supplemented with ferulic acid (100, 500 µM) was on the increase during the root inducing period (day 3-9). Addition of ferulic acid into the rooting medium inhibited root differentiation in its entirety.

On 1.0 µM p-hydroxybenzoic acid containing medium the total peroxidase activity increased continuously between days 0 and 9. The most rapid period of peroxidase development was between days 3 and 6. The specific activity of peroxidase on the same medium increased marginally by day 3. Thereafter the increase was rather sharp and rapid till day 9 (Fig. 104, Table 42).

Table : 36. Growth, Polyphenols and progressive changes in the Activity of Peroxidase, IAA Oxidase, MDH and PAL in Haploid callus tissues of N. tabacum L. var. Anand-2 cultured on root differentiating medium containing 100 uM t-cinnamic acid.

Medium : MS + 2.0 mg/l IAA + 100 uM t-cinnamic acid + 3% sucrose

Incubation : 9 days at 26±2° in continuous light

Inoculum : 300±30 mg fresh tissue

Day	Fresh weight mg/cult.	Dry weight mg/cult.	PEROXIDASE		IAA OXIDASE		MDH		PAL		PHENOLICS	
			units/ cult.	units/mg protein	units/ cult.	units/mg protein	units/ cult.	units/mg protein	units/ cult.	units/mg protein	mg/ cult.	mg%
0	300 (±30)	12 (±3)	0.98	0.86	0.48	0.42	1.05	0.92	0.28	0.25	0.13	1.12
3	501.0 (±20.1)	21.3 (±2.2)	0.70	0.57	1.06	0.86	6.67	5.43	12.24	9.95	0.56	2.63
6	554.2 (±36.3)	40.2 (±3.6)	0.42	0.38	1.17	1.06	3.69	3.33	11.08	10.00	1.37	3.42
9	660.9 (±28.4)	48.0 (±1.9)	0.89	0.41	5.19	2.40	8.92	4.12	32.30	14.93	1.97	4.12

Figures in parenthesis represent standard error.

Data represented is average of 5 replicates.

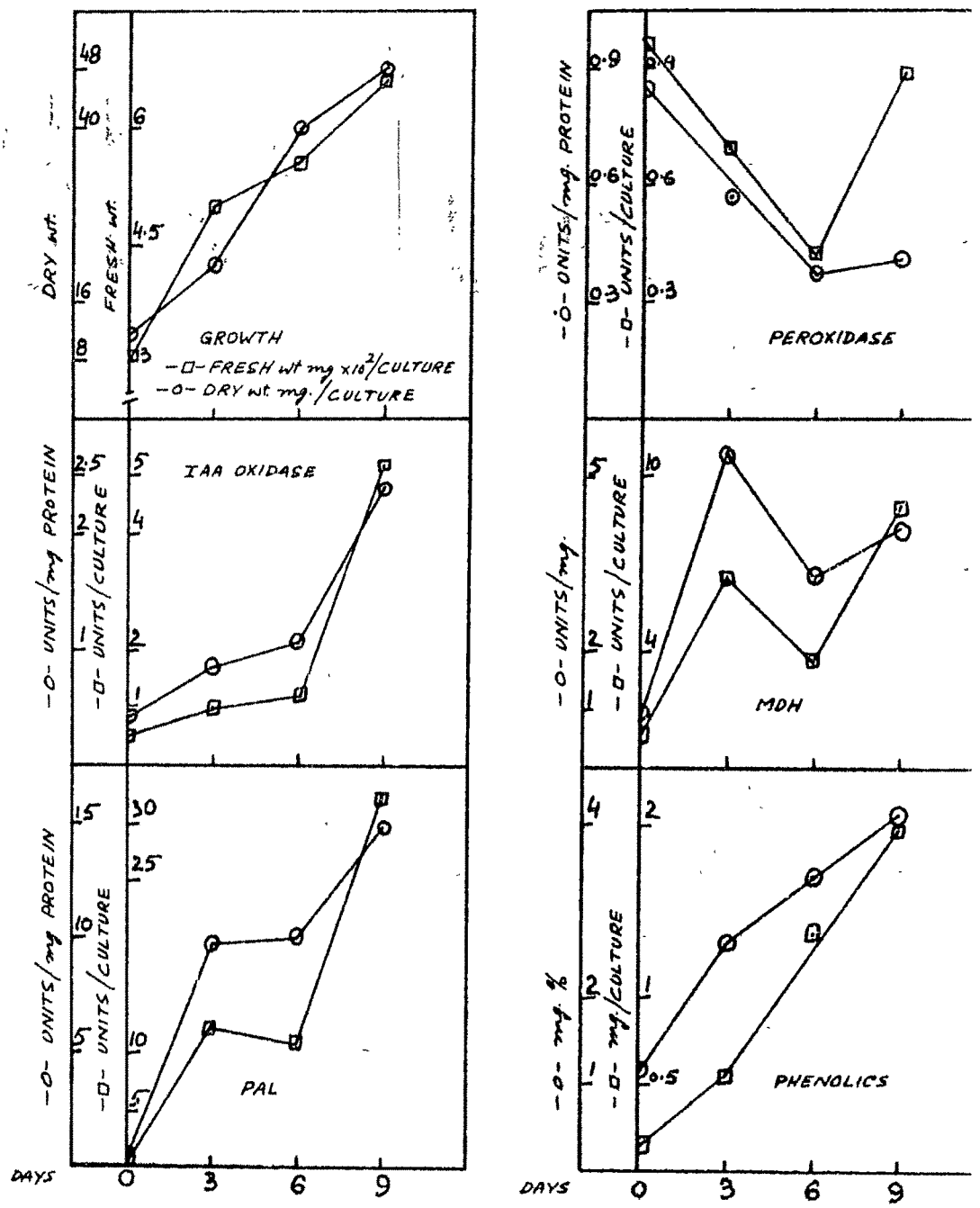
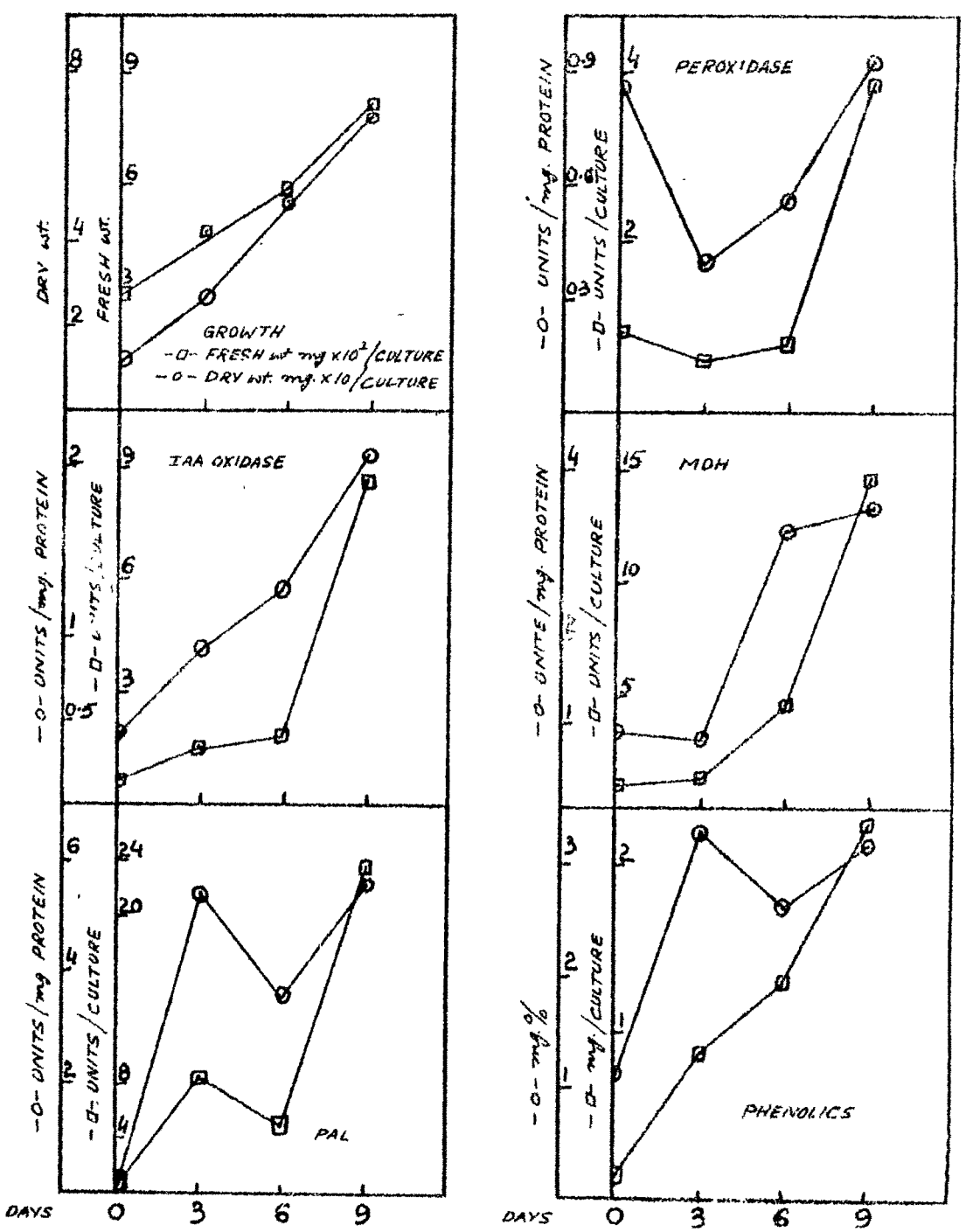


FIG. 9B

Experimental Details as in Table 36.



Experimental Details as in Table 37.

FIG. 99.

Table : 38. Growth, Polyphenols and progressive changes in the Activity of Peroxidase, IAA Oxidase, MDH and PAL in Haploid callus tissues of N. tabacum L. var. Anand-2 cultured on root differentiating medium containing 100 uM caffeic acid.

Medium : MS + 2.0 mg/l IAA + 100 uM caffeic acid + 3% sucrose

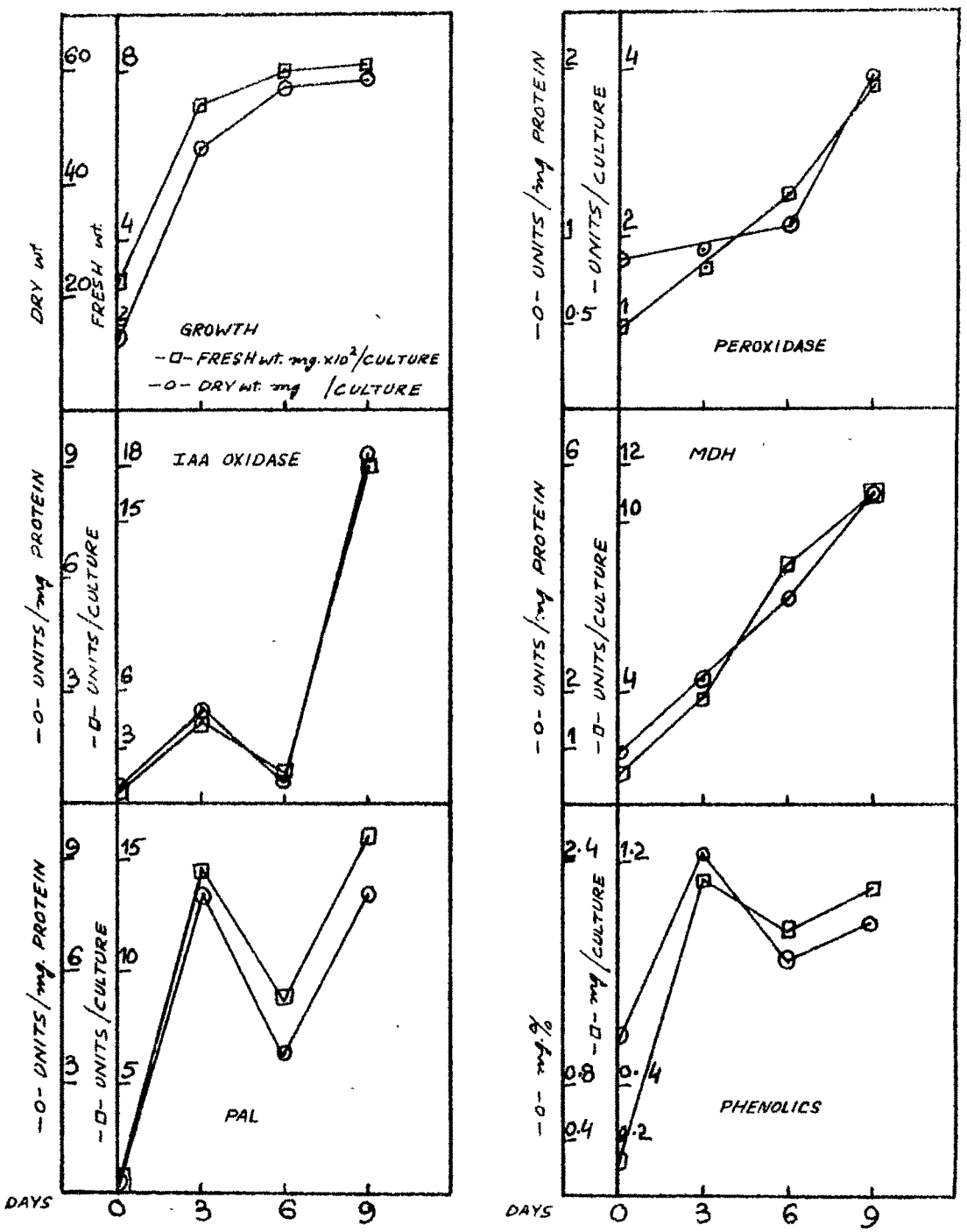
Incubation : 9 days at 26±2° in continuous light

Inoculum : 300±30 mg fresh tissue

Day	Fresh weight mg/cult.	Dry weight mg/cult.	PEROXIDASE		IAA OXIDASE		MDH		PAL		PHENOLICS	
			units/ cult.	units/mg protein	units/ cult.	units/mg protein	units/ cult.	units/mg protein	units/ cult.	units/mg protein	mg/ cult.	mg%
0	300 (±30)	12 (±3)	0.98	0.86	0.48	0.42	1.05	0.92	0.28	0.25	0.13	1.12
3	724.6 (±36.1)	46.5 (±2.2)	1.66	0.93	4.38	2.46	3.86	2.17	14.49	8.14	1.13	2.44
6	806.6 (±40.6)	57.5 (±3.5)	2.52	1.07	1.71	0.72	8.59	3.66	8.96	3.81	0.96	1.68
9	807.7 (±28.3)	58.0 (±5.1)	3.82	1.92	18.21	9.18	10.90	5.50	16.15	8.14	1.13	1.96

Figures in parenthesis represent standard error.

Data represented is average of 5 replicates.



Experimental Details as in Table 38

FIG. 100.

Table : 39. Growth, Polyphenols and progressive changes in the Activity of Peroxidase, IAA Oxidase, MDH and PAL in Haploid callus tissues of N. tabacum L. var. Anand-2 cultured on root differentiating medium containing 500 uM caffeic acid.

Medium : MS + 2.0 mg/l IAA + 500 uM caffeic acid + 3% sucrose

Incubation : 9 days at 26±2° in continuous light

Inoculum : 300±30 mg fresh tissue

Day	Fresh weight mg/cult.	Dry weight mg/cult.	PEROXIDASE		IAA OXIDASE		MDH		PAL		PHENOLICS	
			units/ cult.	units/mg protein	units/ cult.	units/mg protein	units/ cult.	units/mg protein	units/ cult.	units/mg protein	mg/ cult.	mg%
0	300 (±30)	12 (±3)	0.98	0.86	0.48	0.42	1.05	0.92	0.28	0.25	0.13	1.12
3	547.8 (±22.9)	41.2 (±2.4)	0.98	1.10	1.16	1.29	1.45	1.62	8.51	9.50	1.56	3.68
6	869.2 (±43.8)	66.5 (±5.5)	1.79	0.84	2.73	1.28	6.95	3.25	15.44	7.24	2.07	3.12
9	902.9 (±36.3)	75.7 (±5.9)	2.04	0.54	19.29	5.11	8.12	2.15	11.03	2.92	1.61	2.14

Figures in parenthesis represent standard error.

Data represented is average of 5 replicates.

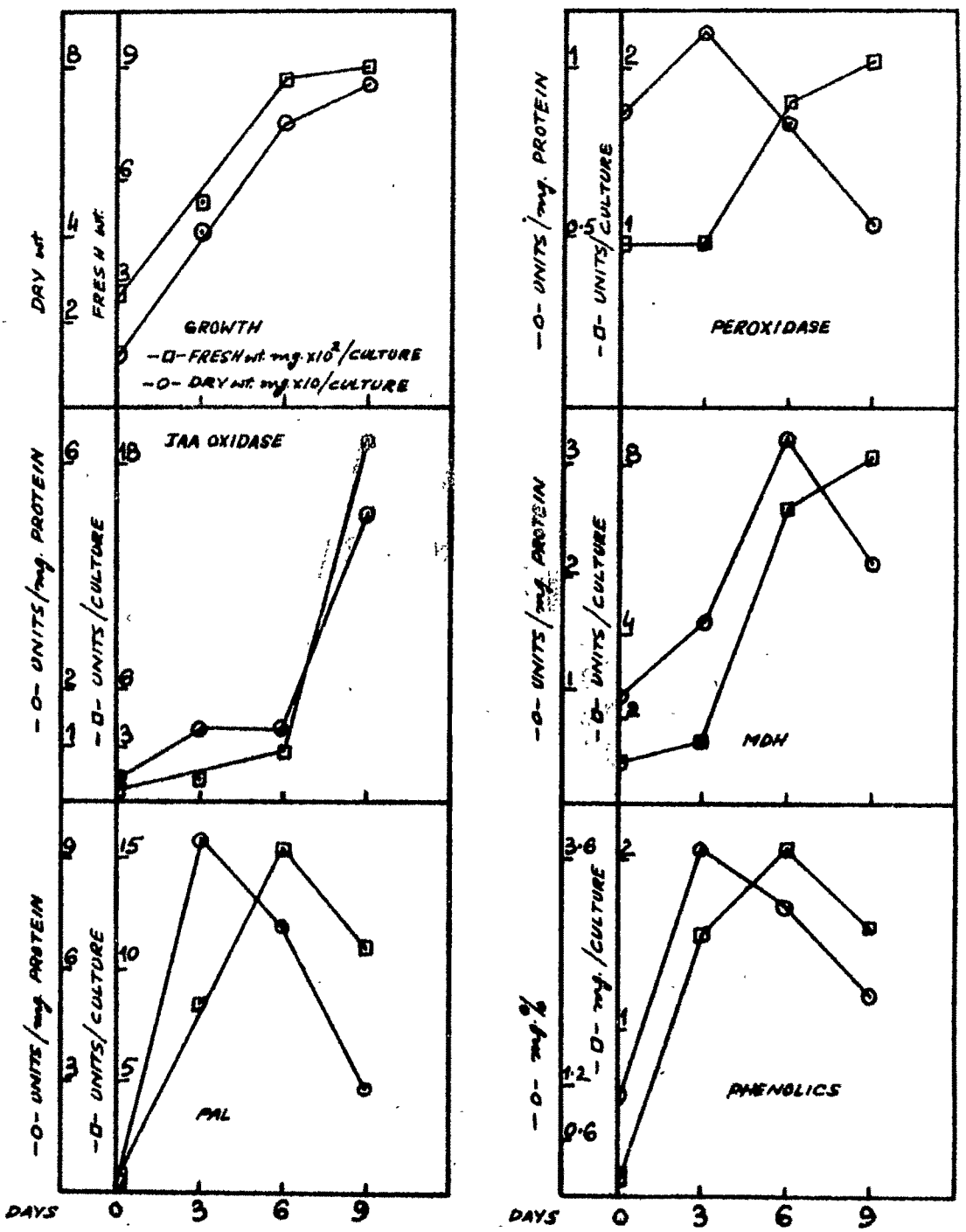


Fig. 101

Experimental Details as in Table 39.

Table : 40. Growth, Polyphenols and progressive changes in the Activity of Peroxidase, IAA Oxidase, MDH and PAL in Haploid callus tissues of N. tabacum L. var. Anand-2 cultured on root differentiating medium containing 100 uM ferulic acid.

Medium : MS + 2.0 mg/l IAA + 100 uM ferulic acid + 3% sucrose

Incubation : 9 days at 26±2° in continuous light

Inoculum : 300±30 mg fresh tissue

Day	Fresh weight mg/cult.	Dry weight mg/cult.	PEROXIDASE		IAA OXIDASE		MDH		PAL		PHENOLICS	
			units/ cult.	units/mg protein	units/ cult.	units/mg protein	units/ cult.	units/mg protein	units/ cult.	units/mg protein	mg/ cult.	mg%
0	300 (±30)	12 (±3)	0.98	0.86	0.48	0.42	1.05	0.92	0.28	0.25	0.13	1.12
3	541.5 (±40.4)	27.0 (±2.8)	1.46	0.82	2.25	1.27	3.24	1.83	11.42	6.45	0.65	2.42
6	697.8 (±41.1)	49.5 (±3.5)	2.80	1.63	2.90	1.69	0.92	0.54	4.64	2.71	0.92	1.87
9	727.9 (±36.8)	51.7 (±4.1)	3.48	2.92	8.69	7.30	13.10	11.00	21.02	17.65	1.79	3.48

Figures in parenthesis represent standard error.

Data represented is average of 5 replicates.

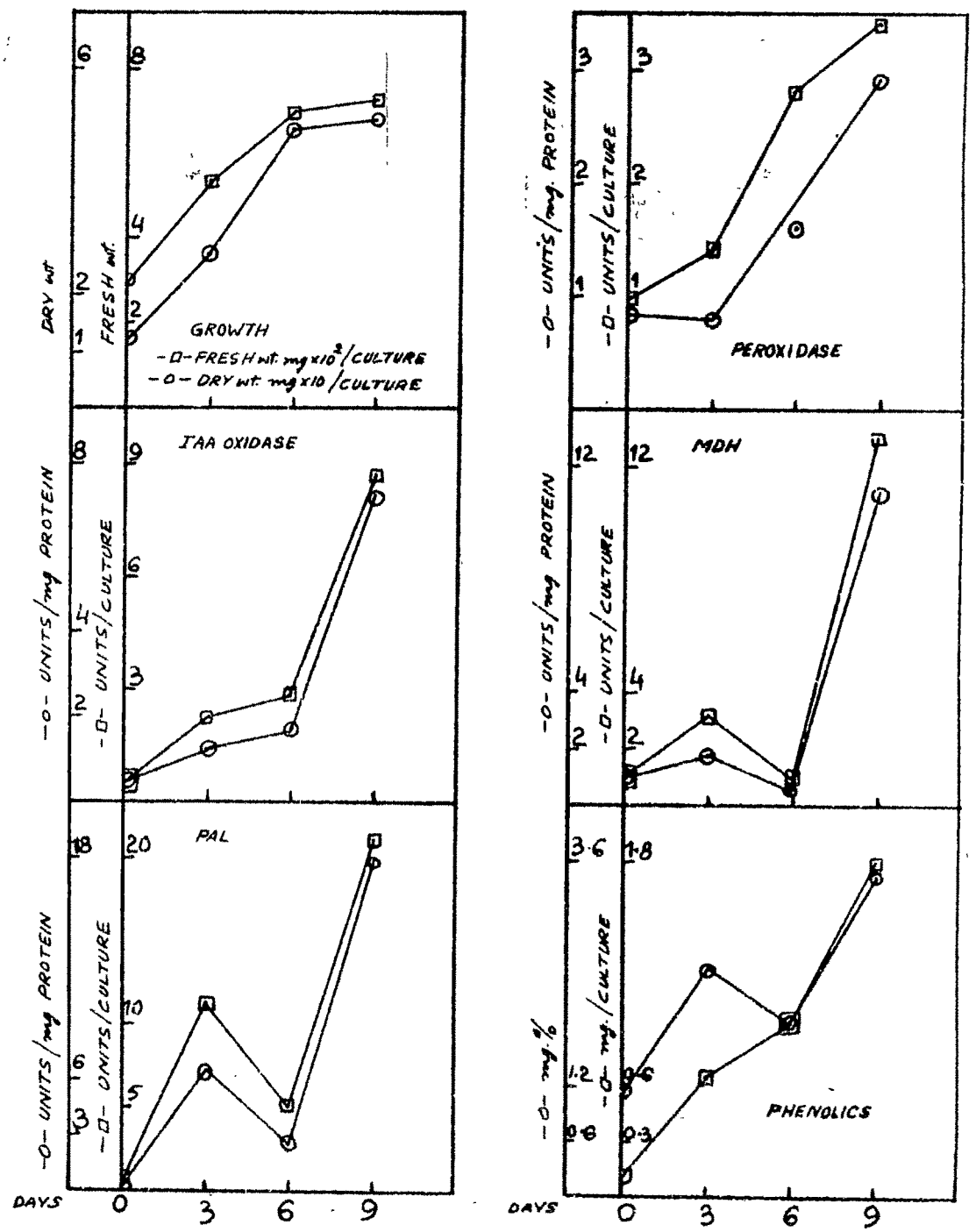


Fig. 102.

Experimental Details as in Table 40

Table : 41. Growth, Polyphenols and progressive changes in the Activity of Peroxidase, IAA Oxidase, MDH and PAL in Haploid callus tissues of N. tabacum L. var. Anand-2 cultured on root differentiating medium containing 500 uM ferulic acid.

Medium : MS + 2.0 mg/l IAA + 500 uM ferulic acid + 3% sucrose

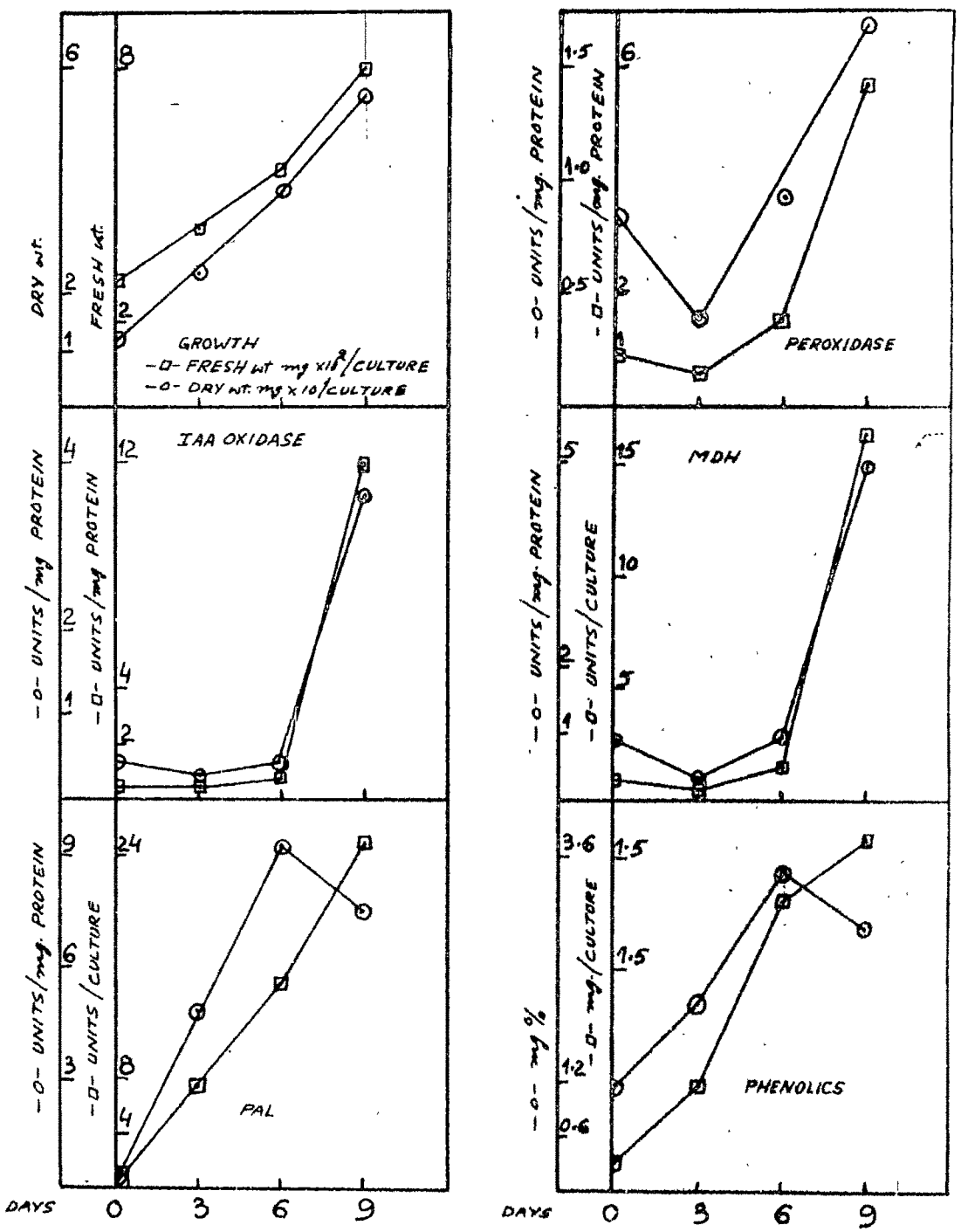
Incubation : 9 days at 26±2° in continuous light

Inoculum : 300±30 mg fresh tissue

Day	Fresh weight mg/cult.	Dry weight mg/cult.	PEROXIDASE		IAA OXIDASE		MDH		PAL		PHENOLICS	
			units/ cult.	units/mg protein	units/ cult.	units/mg protein	units/ cult.	units/mg protein	units/ cult.	units/mg protein	mg/ cult.	mg%
0	300 (±30)	12 (±3)	0.98	0.86	0.48	0.42	1.05	0.92	0.28	0.25	0.13	1.12
3	424.3 (±28.6)	23.9 (±2.3)	0.59	0.37	0.46	0.29	0.56	0.35	7.53	4.76	0.48	2.04
6	556.9 (±33.8)	38.3 (±3.6)	1.51	0.93	0.61	0.37	1.48	0.91	14.84	9.16	1.30	3.42
9	798.6 (±42.6)	55.0 (±4.7)	5.60	1.67	11.92	3.57	16.37	4.90	24.84	7.44	1.57	2.86

Figures in parenthesis represent standard error.

Data represented is average of 5 replicates.



Experimental Details as in Table 41.

Fig. 103.

Table : 42. Growth, Polyphenols and progressive changes in the Activity of Peroxidase, IAA Oxidase, MDH and PAL in Haploid callus tissues of N. tabacum L. var. Anand-2 cultured on root differentiating medium containing 1.0 uM p-hydroxybenzoic acid.

Medium : MS + 2.0 mg/l IAA + 1.0 uM p-hydroxybenzoic acid + 3% sucrose

Incubation : 9 days at 26±2° in continuous light

Inoculum : 300±30 mg fresh tissue

Day	Fresh weight mg/cult.	Dry weight mg/cult.	PEROXIDASE		IAA OXIDASE		MDH		PAL		PHENOLICS	
			units/ cult.	units/mg protein	units/ cult.	units/mg protein	units/ cult.	units/mg protein	units/ cult.	units/mg protein	mg/ cult.	mg%
0	300 (±30)	12 (±3)	0.98	0.86	0.48	0.42	1.05	0.92	0.28	0.25	0.13	1.12
3	492.4 (±33.6)	23.3 (±2.1)	1.87	0.90	3.86	1.87	1.30	0.63	18.59	9.03	0.84	3.62
6	941.8 (±48.2)	74.2 (±4.7)	5.22	1.69	2.96	0.96	10.03	3.25	20.92	6.79	2.08	2.81
9	998.6 (±58.5)	78.9 (±6.7)	6.64	2.03	14.91	4.56	22.96	7.02	17.74	5.43	1.67	2.12

Figures in parenthesis represent standard error.

Data represented is average of 5 replicates.

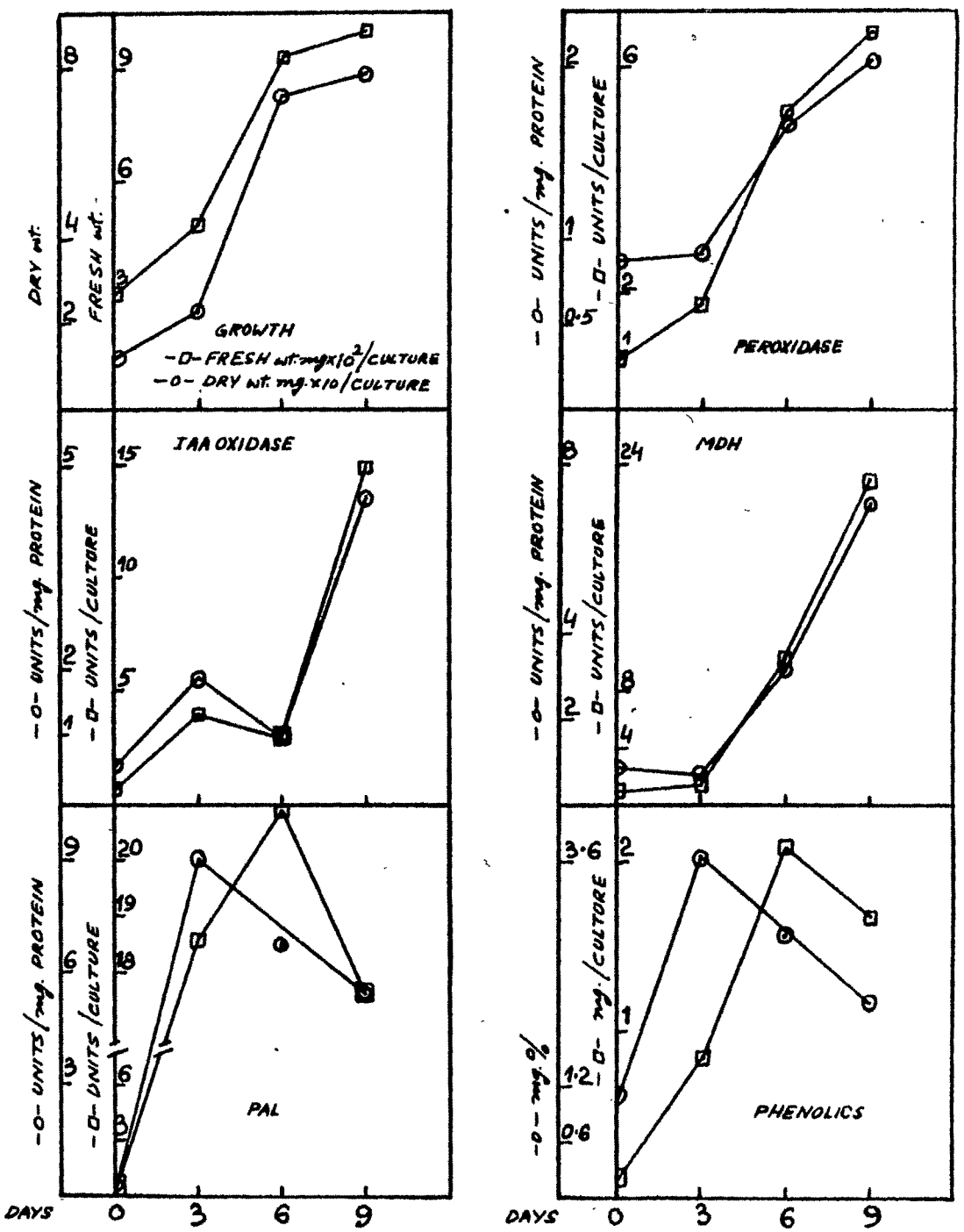


FIG. 104.

Experimental Details as in Table 42.

Table : 43. Growth, Polyphenols and progressive changes in the Activity of Peroxidase, IAA Oxidase, MDH and PAL in Haploid callus tissues of N. tabacum L. var. Anand-2 cultured on root differentiating medium containing 10.0 uM p-hydroxybenzoic acid.

Medium : MS + 2.0 mg/l IAA + 10.0 uM p-hydroxybenzoic acid + 3% sucrose

Incubation : 9 days at 26±2° in continuous light

Inoculum : 300±30 mg fresh tissue

Day	Fresh weight mg/cult.	Dry weight mg/cult.	PEROXIDASE		IAA OXIDASE		MDH		PAL		PHENOLICS	
			units/ cult.	units/mg protein	units/ cult.	units/mg protein	units/ cult.	units/mg protein	units/ cult.	units/mg protein	mg/ cult.	mg%
0	300 (±30)	12 (±3)	0.98	0.86	0.48	0.42	1.05	0.92	0.28	0.25	0.13	1.12
3	620.5 (±46.2)	29.1 (±2.3)	1.11	0.48	3.75	1.62	0.82	0.35	28.95	12.51	1.00	3.46
6	769.4 (±54.9)	52.3 (±4.2)	3.59	2.33	1.62	1.05	10.25	6.66	20.51	13.33	1.89	3.63
9	1008.3 (±82.8)	68.6 (±4.8)	10.53	3.59	12.83	4.37	20.67	7.04	34.72	11.84	2.14	3.12

Figures in parenthesis represent standard error.

Data represented is average of 5 replicates.

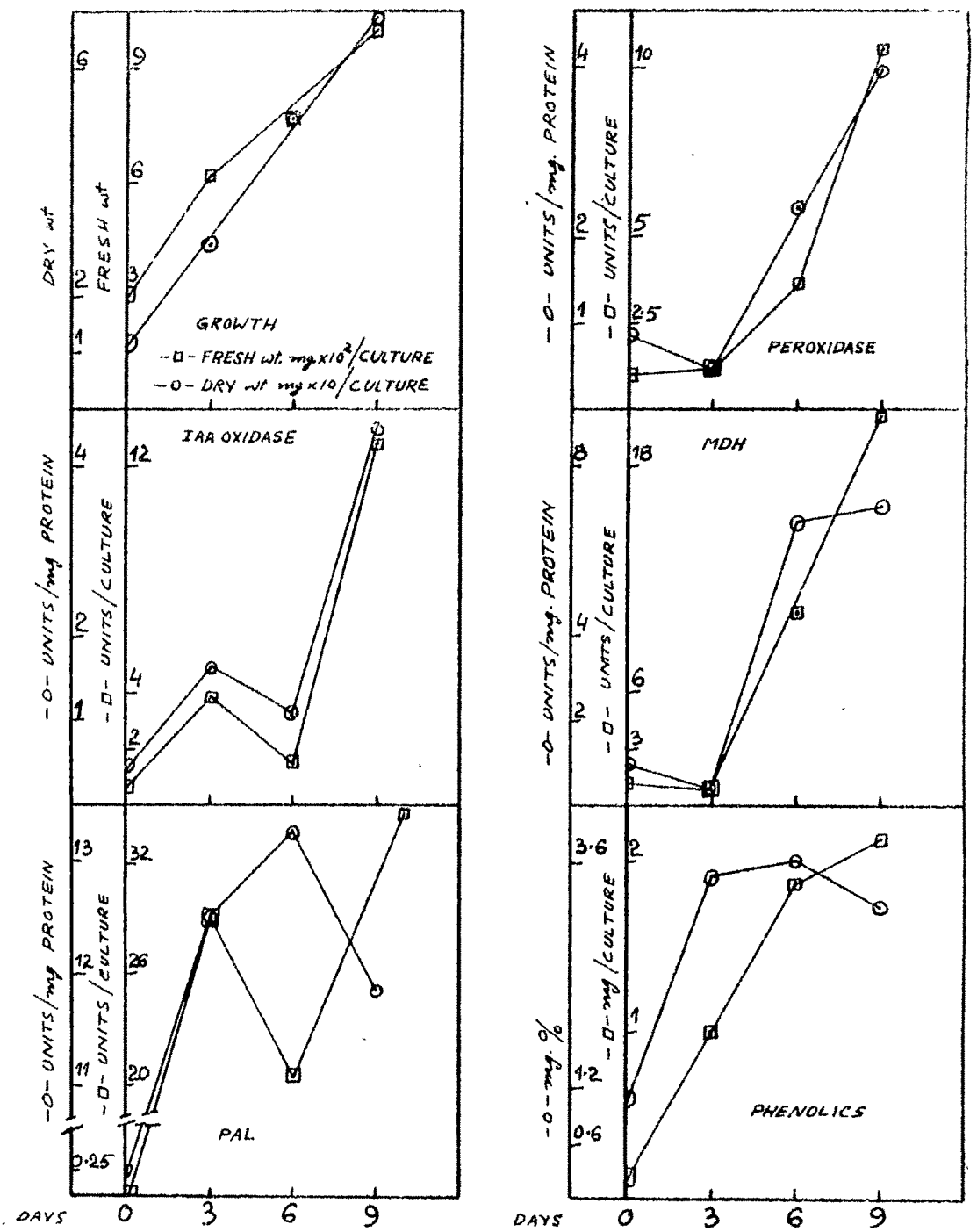


FIG. 105

Experimental Details as in Table 43.

The total peroxidase activity increased modestly between day 0-3 in haploid tobacco callus cultured on 10.0 µM p-hydroxybenzoic acid containing medium. Between days 3 and 9 the activity, however, increased very rapidly. The specific peroxidase activity declined during the initial 3 days in culture, but thereof it shot up along a linear gradient to its peak value by day 9 (Fig. 105, Table 43).

Incorporation of p-hydroxybenzoic acid (1.0, 10.0 µM) into the medium resulted in the increase of peroxidase activity per unit protein during the period of root differentiation (days 3-9). Supplementation of p-hydroxybenzoic acid into the root inducing medium brought about total inhibition of root differentiation.

(c) IAA Oxidase :

The progressive changes in IAA Oxidase activity in haploid tobacco callus cultured on root inducing medium (MS + 2.0 mg/l IAA + 3% sucrose) supplemented with different phenolic acids, are illustrated in Figs. 98 - 105 and Tables 36 - 43.

Both total and specific activities of IAA Oxidase in haploid callus cultured on 100 µM t-cinnamic acid containing medium exhibited identical developmental pattern. The enzyme activity increased at a slow rate between days 0 and 6.

Thereafter they increased sharply to attain peak values on day 9 (Fig. 98, Table 36). On 500 µM t-cinnamic acid total IAA Oxidase activity increased rather slowly till day 6, but thereof the increase till day 9 was very sharp. On the other hand, the specific activity increased rapidly from day 0 till day 9 (Fig. 99, Table 37).

Incorporation of t-cinnamic acid (100, 500 µM) into the root inducing medium resulted in increase of IAA Oxidase activity per unit protein during the period of root differentiation (day 3-9). The enzyme activity on day 6 was at least 30 per cent more in callus tissues cultured on 500 µM t-cinnamic acid medium which inhibited root differentiation, than in callus tissues cultured on 100 µM t-cinnamic acid medium. Roots were differentiated at the lower level of t-cinnamic acid, whereas the higher level inhibited it.

The total and specific activity of IAA Oxidase in haploid tobacco tissues cultured on 100 µM caffeic acid containing medium, demonstrated double peaked pattern of development. The first peak value was attained on day 3. The second peak value was reached on day 9 following a sharp increase in the activity (Fig. 100, Table 38). On 500 µM caffeic acid containing medium the total IAA Oxidase activity increased slowly till day 6. A sharp increase in the activity ensued thereafter reaching its peak value on day 9. The specific

IAA Oxidase activity on the same medium increased till day 3 and then declined marginally by day 6. Thereof, the activity increased sharply to reach its peak value on day 9 (Fig. 101, Table 39).

The IAA Oxidase activity per unit protein during root differentiation from haploid callus tissues cultured on 500 µM caffeic acid containing root inducing medium remained stable at a level between days 3 and 6. The roots were differentiated on day 6. Thereafter, the activity increased. On the other hand, on 100 µM caffeic acid medium, which inhibited root differentiation the enzyme activity declined sharply during the corresponding period of culture.

Both total and specific activities of IAA Oxidase followed identical patterns of development in haploid tobacco tissues cultured on 100 µM ferulic acid containing medium. The activity till day 6 increased rather slowly; thereafter, sharp increase in the activities ensued. The peak values were reached on day 9 (Fig. 102, Table 40). On 500 µM ferulic acid containing medium the total and specific IAA Oxidase activities declined slightly during the first 3 days. By day 6 the activities had, however, registered a modest increase. After day 6 the activities shot up to the peak values on day 9 (Fig. 103, Table 41).

Incorporation of ferulic acid (100, 500 µM) into the

medium inhibited root differentiation. The IAA Oxidase activity per unit protein during the root inducing period (day 3-9) was on the increase on both the above mentioned media.

Both total and specific IAA Oxidase activities in haploid callus tissues demonstrated double peaked developmental pattern when cultured on medium containing 1.0 and 10.0 µM p-hydroxy-benzoic acid. On both the media the first peak value was reached on day 3 and the second on day 9 (Figs. 104, 105; Tables 42, 43).

p-Hydroxybenzoic acid (1.0, 10.0 µM) brought about inhibition of root differentiation. The IAA Oxidase activity per unit protein declined during the days immediately preceding the normal period of root differentiation (days 3-6) and then increased sharply between days 6-9.

(d) MDH :

The progressive changes in MDH activity in haploid tobacco callus tissues cultured on rooting medium supplemented with phenolic acids individually, are illustrated in Figs. 98 - 105, and Tables 36 - 43.

The total and specific MDH activity in tissue cultured on medium containing 100 µM t-cinnamic acid, demonstrated double

peaked developmental pattern. The first peak value was reached on day 3 and the second on day 9 (Fig. 98, Table 36). On 500 µM t-cinnamic acid containing medium the increase in total MDH activity between days 0 and 3 was very marginal. Thereafter the increase in the activity was rather sharp till day 9, when the peak value was reached. The specific MDH activity, on the other hand, declined slightly by day 3, followed by a sharp increase till day 6. The activity continued to increase thereafter rather slowly till day 9 (Fig. 99, Table 37).

The MDH activity per unit protein was on the decline during days immediately preceding root differentiation (days 3-6) in haploid callus cultured on root inducing medium supplemented with 100 µM t-cinnamic acid, whereas the inverse trend was followed when 500 µM t-cinnamic acid was used. Furthermore, at the higher level of t-cinnamic acid root differentiation was inhibited.

On 100 µM caffeic acid containing medium the total and specific activities of MDH were on increase from day 0 till day 9, when the peak values were attained (Fig. 100, Table 38). On 500 µM caffeic acid medium the total MDH activity registered a slight increase during the initial 3 days. Thereafter the activity increased to its peak value on day 9. On the contrary, the specific activity increased between days 0 and 6 to reach

the peak value. Thereafter the activity declined (Fig. 101, Table 39).

The MDH activity per unit protein was an increase during the days immediately preceding root differentiation (days 3-6) in haploid tobacco tissues cultured on root inducing medium supplemented with 500 μM caffeic acid. Same trend was followed by the development of enzyme activity when 100 μM caffeic acid was incorporated into the medium. The low level of caffeic acid inhibited root differentiation whereas the higher one induced it.

The total and specific activities of MDH demonstrated double peaked pattern of development in haploid tobacco callus cultured on 100 μM ferulic acid. The first peak value was attained on day 3 and the second on day 9 (Fig. 102, Table 40). On 500 μM ferulic acid medium both, total and specific MDH activities declined slightly during the first 3 days. By day 6 it had, however, increased again but only marginally. Between days 6 and 9 the activities increased very sharply to peak values on day 9 (Fig. 103, Table 41).

The incorporation of ferulic acid (100, 500 μM) into the root inducing medium brought about total inhibition of root differentiation from haploid tobacco callus tissues. The MDH activity per unit protein in callus tissues cultured on 100 μM ferulic acid medium was on the decline between days 3-6, whereas

on 500 µM ferulic acid medium it was on the increase. Between days 6-9 the enzyme activity increased sharply on both the media.

The total activity of MDH in haploid callus cultured on 1.0 µM p-hydroxybenzoic acid containing medium registered only a marginal increase by day 3. Thereafter it increased very sharply and almost linearly till day 9 to its peak value. On the other hand, the specific activity of MDH on the same medium decreased slightly by day 3, only to register a sharp and linear increase thereof till day 9, attaining its peak value (Fig. 104, Table 42). On medium containing 10.0 µM p-hydroxybenzoic acid the total MDH activity decayed slightly during the first 3 days of culture. Thereof it increased sharply and linearly to its peak value on day 9. The specific activity of MDH also demonstrated a slight decline in the activity during the first 3 days in culture, followed by sharp increase in the activity till day 6. The activity continued to increase till day 9, but the increase between days 6 and 9 was rather modest (Fig. 105, Table 43).

The MDH activity per unit protein of tobacco callus cultured on medium supplemented with 1.0 and 10.0 µM p-hydroxybenzoic acid, increased between days 3-9, reaching almost identical values on day 9. On both these media the root differentiation was inhibited.

(e) PAL :

The progressive changes in the total and specific activities of PAL in haploid tobacco callus cultured on root inducing medium supplemented with various phenolic acids individually, are illustrated in Figs. 98 - 105, and Tables 36 - 43.

The total PAL activity in tissues cultured on 100 μM of t-cinnamic acid increased rather sharply between days 0 and 3. By day 6 it, however, declined slightly. Thereof, the activity increased again steeply to its peak value on day 9. The specific activity of PAL also increased sharply between days 0 and 3. The increase in the activity was marginall between days 3 and 6, but thereof, the activity increased again rapidly to attain the peak value on day 9 (Fig. 98, Table 36). On 500 μM t-cinnamic acid medium the PAL activities, total and specific, demonstrated double peaked developmental pattern. The first peak was attained on day 3 and the second on day 9 (Fig. 99, Table 37).

The PAL activity per unit protein of haploid tissues remained more or less stable at a level between days 3-6 (the period preceding root differentiation) on root inducing medium supplemented with 100 μM t-cinnamic acid. With the incorporation of 500 μM t-cinnamic acid into the rooting

medium the enzyme activity was on the decline between days 3-6, and also root differentiation was inhibited on this medium.

The total and specific PAL activity in haploid tissues cultured on 100 μM caffeic acid medium reached two peak values during the culture period. The first peak value was reached on day 3 and the second on day 9 (Fig. 100, Table 38). On 500 μM caffeic acid medium the total PAL activity increased rapidly and linearly to its peak value on day 6. Thereafter, the enzyme activity declined. The specific activity of PAL reached its peak value on day 3 following sharp increase in the activity. Thereof till day 9, the specific PAL activity decayed rapidly (Fig. 101, Table 39).

The PAL activity per unit protein in haploid tobacco tissues was on decline between days 3-6 and on increase between days 6-9 on root inducing medium supplemented with 100 μM caffeic acid. On incorporation of 500 μM caffeic acid into the rooting medium the enzyme activity was on decline between days 3-6 and 6-9 i.e., the days preceding and following root differentiation. The lower concentration of caffeic acid (i.e. 100 μM) inhibited root differentiation.

On 100 μM ferulic acid containing medium the total and specific activities of PAL showed identical developmental

pattern. The activities attained two peak values, one each on days 3 and 9, during the culture period (Fig. 102, Table 40). On 500 µM ferulic acid medium the total PAL activity increased rapidly and almost linearly between days 0 and 9. The peak value was reached on day 9. The specific activity, on the other hand, reached its peak value on day 6 following sharp and linear increase in the activity. Thereafter, by day 9 the activity declined (Fig. 103, Table 41).

The PAL activity per unit protein of haploid tobacco callus was on the decline between days 3-6 and on the increase between days 6-9, when 100 µM ferulic acid was incorporated into the rooting medium. Exactly inverse trend was followed by enzyme development with incorporation of 500 µM ferulic acid into the medium. Both levels of ferulic acid, however, inhibited root differentiation.

The total PAL activity in haploid tobacco callus cultured on medium supplemented with 1.0 µM p-hydroxybenzoic acid increased very sharply till day 6, reaching its peak value. It, however, decayed thereafter till day 9. The specific PAL activity reached its peak value on day 3 as a consequence of rapid increase in the activity. Between days 3 and 9 the activity was on decline (Fig. 104, Table 42). On 10.0 µM p-hydroxybenzoic acid medium the total and specific PAL activities were much higher than in the previous case. Further,

the total PAL activity demonstrated double peaked developmental pattern. The first peak value was attained on day 3 and the second on day 9. The specific PAL activity, on the other hand, continued to increase rapidly till day 6 reaching its peak value. Thereafter, the activity declined (Fig. 105, Table 43).

On incorporation of 1.0 μM p-hydroxybenzoic acid into the root inducing medium the PAL activity per unit protein in haploid tobacco tissues was on the decline between days 3-9 (i.e., the period preceding root differentiation and after it). On using 10.0 μM p-hydroxybenzoic acid in the medium the enzyme activity was on increase between days 3-6 and declined between days 6-9. Incorporation of p-hydroxybenzoic acid into the medium brought about total inhibition of root differentiation from haploid callus tissues.

(f) Phenolics :

The progressive changes in the accumulation of phenolics in haploid tobacco callus cultured on root inducing medium supplemented with various phenolic acids individually, are illustrated in Figs. 98-105, and Tables 36-43.

The phenolic accumulation was on increase right from the beginning in haploid callus cultured on 100 μM t-cinnamic acid medium. The phenolics increased from 0.13 mg/culture on day 0 to 1.97 mg/culture on day 9. On percentage basis the

phenolic content increased from 1.12 mg% on day 0 to 4.12 mg% on day 9 (Fig. 98, Table 36). On 500 μM t-cinnamic acid medium the phenolic content increased from 0.13 mg/culture on day 0 to 2.23 mg/culture on day 9. On percentage basis the phenolic content increased from 1.12 mg% on day 0 to 3.28 mg% on day 3. By day 6 it declined to 2.62 mg%. But by day 9 it had again increased to 3.18 mg% (Fig. 99, Table 37).

On 100 μM t-cinnamic acid containing root inducing medium the phenolic content in haploid callus tissues increased from 2.63 mg% to 3.42 mg% between days 3-6 i.e., immediately preceding root differentiation. On 500 μM t-cinnamic acid containing medium, whereon root differentiation was completely inhibited the phenolic content decreased from 3.28 mg% to 2.62 mg% between days 3-6 and increased from 2.62 mg% to 3.18 mg% between days 6-9.

On 100 μM caffeic acid containing medium the phenolic content increased from 0.13 mg/culture to 1.13 mg/culture by day 3. By day 6 it, however, declined to 0.96 mg/culture, only to increase again to 1.13 mg/culture on day 9. On percentage basis the phenolics increased from 1.12 mg% to 2.44 mg% by day 3. By day 6 it declined to 1.68 mg%, but recovered again to reach 1.96 mg% on day 9 (Fig. 100, Table 38). The phenolics in haploid tobacco callus tissues cultured on 500 μM caffeic acid medium increased, from

0.13 mg/culture to 2.07 mg/culture by day 6, but dropped to 1.61 mg/culture by day 9. On percentage basis, the phenolics increased from 1.12 mg% to 3.68 mg% by day 3. Thereafter, it declined to 2.14 mg% on day 9 (Fig. 101, Table 39).

On 100 μM caffeic acid containing root inducing medium the phenolic content declined between days 3-6, from 2.44 mg% to 1.68 mg%. Thereof it, however, increased to 1.96 mg% by day 9. On 500 μM caffeic acid containing medium the phenolic content was 3.68 mg% before root differentiation on day 3, 3.12 mg% on day 6 i.e.,the day of root differentiation and 2.14 mg% on day 9 i.e., the days following root differentiation.

On 100 μM ferulic acid containing medium the phenolic content increased between days 0 and 9. It increased from 0.13 mg/culture on day 0 to 1.79 mg/culture on day 9. On percentage basis the phenolics increased from 1.12 mg% to 2.42 mg% by day 3, but dropped again to 1.87 mg% by day 6. The phenolic accumulation, however, reached its peak value of 3.48 mg% on day 9 (Fig. 102, Table 40). On 500 μM ferulic acid medium the phenolic accumulation on culture basis was on the increase right through the culture period, increasing from 0.13 mg/culture on day 0 to 1.57 mg/culture on day 9. On percentage basis the phenolic accumulation reached its peak value of 3.42 mg% on day 6. It then declined to 2.86 mg% by day 9 (Fig. 103, Table 41).

Incorporation of ferulic acid (100, 500 µM) into the root inducing medium resulted in total inhibition of root differentiation from haploid callus tissues. The phenolic content on 100 µM ferulic acid medium was on the decline between days 3-6, and increased thereafter. On 500 µM ferulic acid medium the phenolic content increased between days 3-6 and decreased thereof.

In haploid tobacco callus tissues cultured on 1.0 µM p-hydroxybenzoic acid containing medium the peak value of 2.08 mg/culture of phenolic accumulation was attained on day 6, thereafter, the content declined to 1.67 mg/culture on day 9. On percentage basis the peak value of 3.62 mg% was reached on day 3. It then declined to 2.12 mg% by day 9 (Fig. 104, Table 42). On 10.0 µM p-hydroxybenzoic acid containing medium the phenolic accumulation on culture basis was continuously on the increase right through the culture period. It increased from 0.13 mg/culture to 2.14 mg/culture on day 9. On percentage basis the peak value of 3.63 mg% was attained on day 6. By day 9 it had declined to 3.12 mg% (Fig. 105, Table 43).

On incorporation of 1.0 µM p-hydroxybenzoic acid in medium the phenolic content in haploid callus tissues was on decline between days 3-9, whereas on 10.0 µM p-hydroxybenzoic acid medium it increased between days 3-6 and declined between

days 6-9. Both the levels of p-hydroxybenzoic acid in the root inducing medium inhibited root differentiation.

(g) Peroxidase Isoenzymes :

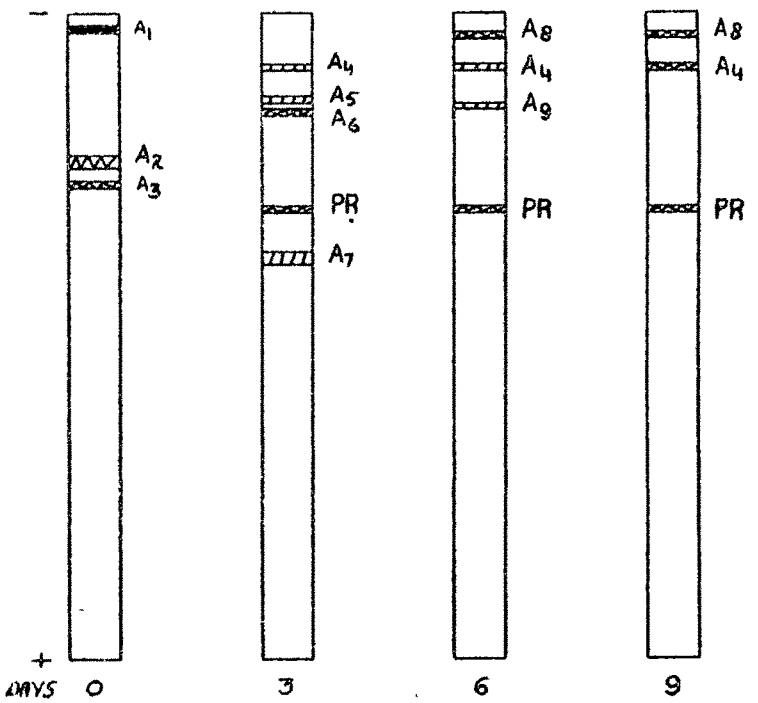
Progressive changes in the anodic and cathodic banding patterns of peroxidase isoenzymes in haploid tobacco callus cultures on MS medium supplemented with 2.0 mg/l IAA and various phenolic acids are illustrated in Figs. 98a - 105a.

With incorporation of 100 μM t-cinnamic acid into the root inducing medium, original three anodic isoperoxidases A_1, A_2 and A_3 remained suppressed throughout the culture period. Six slow migrating anodic isoperoxidases A_4-A_9 were synthesized at different intervals during the culture period. Anodic isoperoxidase A_4 appeared on days 3, 6 and 9; A_5, A_6 and A_7 on day 3; A_8 on days 6 and 9, and A_9 on day 6. Root differentiation in haploid tobacco callus tissues cultured on the above medium was preceded by the appearance of anodic isoperoxidase "PR", which was also detected in peroxidase preparations of regenerated roots. Only C_2 and C_3, of the three initial cathodic isoperoxidases were suppressed during the culture period. C_1 appeared on all days. A slow migrating cathodic isoperoxidase C_4 was synthesized on days 3 and 6. (Fig. 98a).

The increase of t-cinnamic acid to 500 μM in the above

medium, resulted in suppression of three initial anodic isoperoxidases A_1, A_2 and A_3. Root peroxidase "PR" and root differentiation were also suppressed. During the 9 day culture period, seven slow migrating anodic isoperoxidases were synthesized. Of these A_4 and A_5 appeared on day 3; A_6 on days 3 and 6; A_7 and A_8 on days 6 and 9; A_9 and A_{10} on day 9. Only one original cathodic isoperoxidase C_1 appeared on all days of culture, out of the three initial ones. A slow migrating cathodic isoperoxidase C_4 appeared on days 3, 6 and 9. Another slow migrating one C_5 appeared only on day 3 (Fig. 99a).

On the culture of haploid tobacco callus tissues on 100 μM caffeic acid containing medium, initial anodic isoperoxidases A_1, A_2 and A_3 did not make appearance at any time during the culture period. The root peroxidase "PR" and differentiation of roots was also suppressed. During the culture period six slow migrating anodic isoperoxidases were synthesized. Of these A_4 and A_6 were synthesized on day 3; A_5 on days 3, 6 and 9; A_7 and A_8 on days 6 and 9; and A_9 on day 9. Of the three initial cathodic isoperoxidases, only C_1 appeared on all days of culture. The other two C_2 and C_3, remained suppressed right through. One slow migrating cathodic isoperoxidase, C_4, appeared on days 3 and 6 (Fig. 100a).



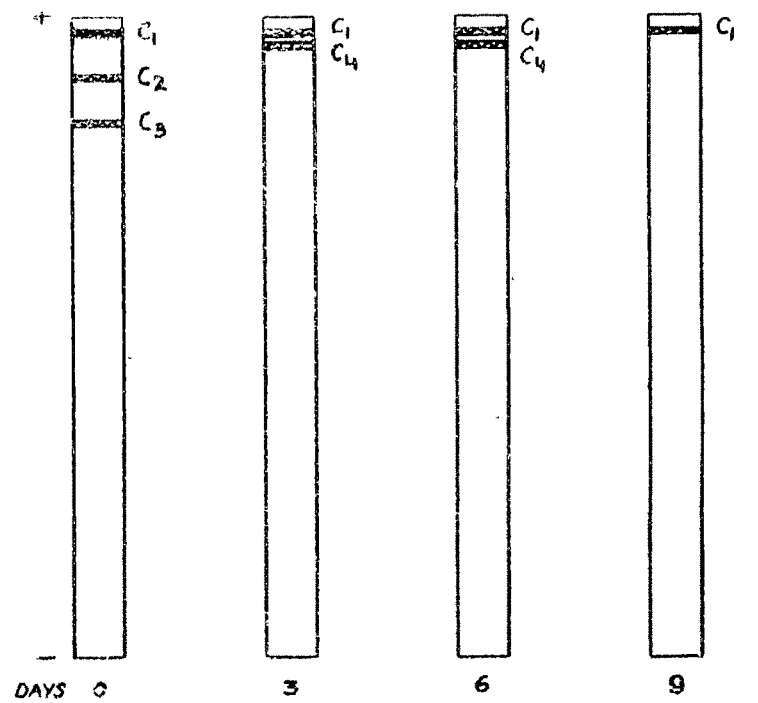


Fig. 98a.

Anodic and Cathodic Isoperoxidase Patterns

Experimental Details as in Table 36.

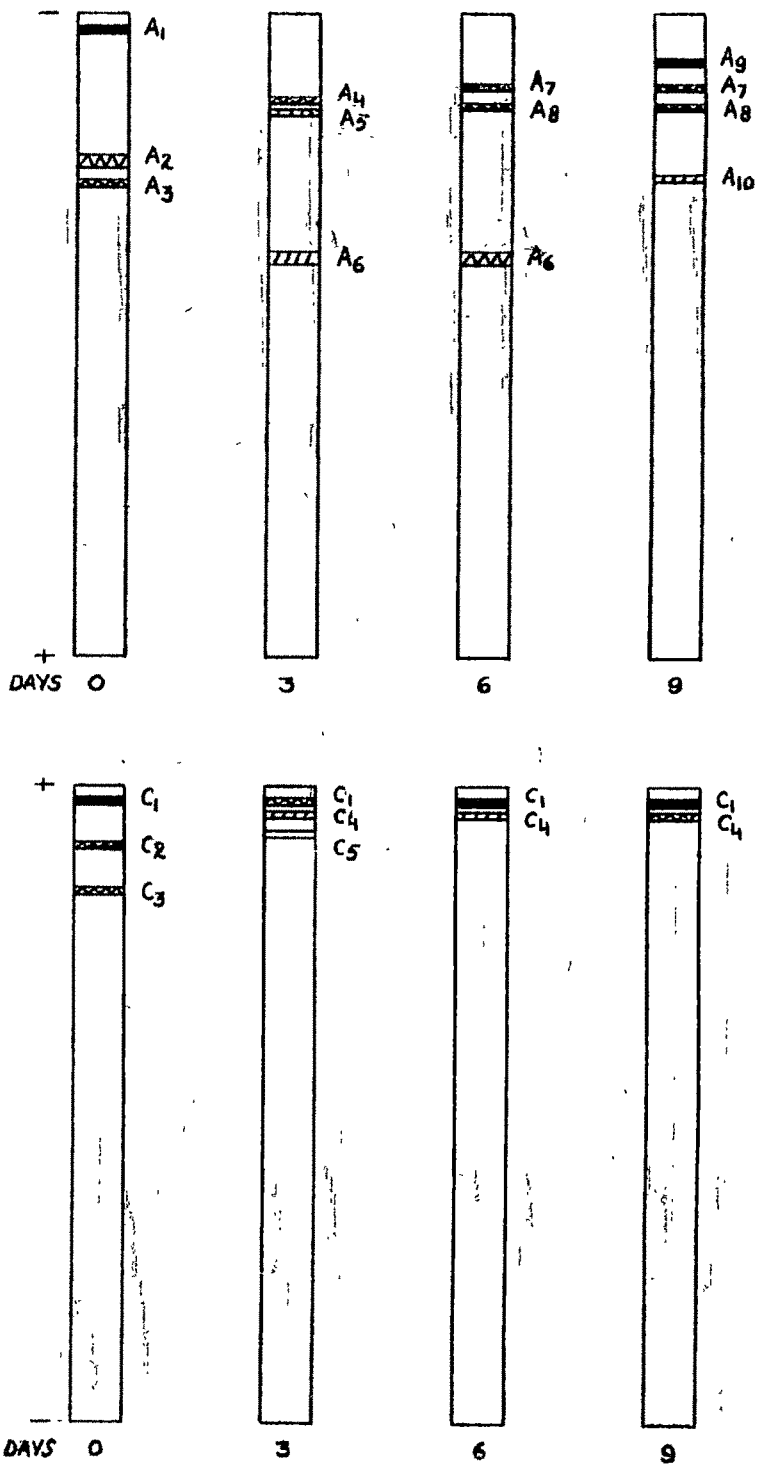


FIG. 99a

Anodic and Cathodic Isoperoxidas Patterns.

Experimental Details as in Table 37.

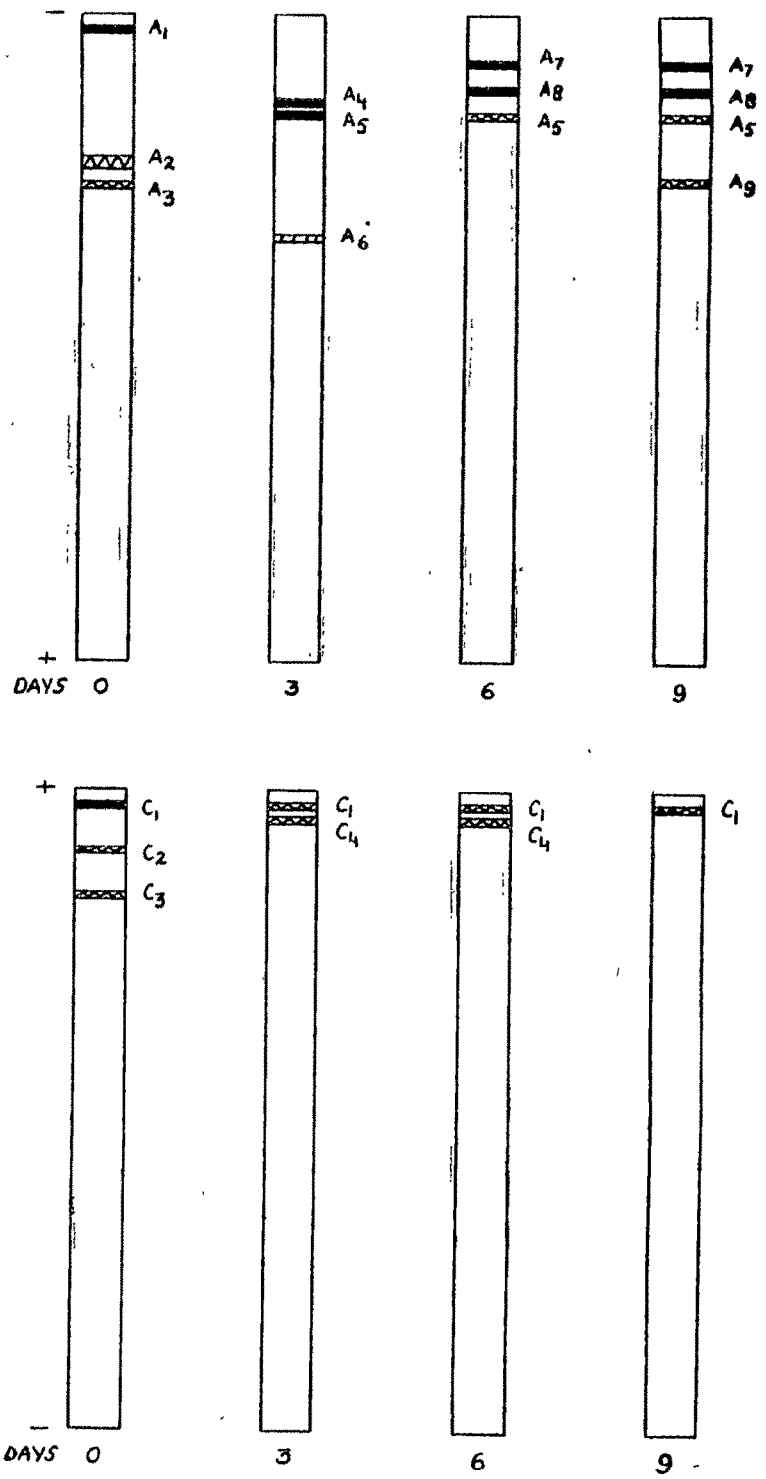
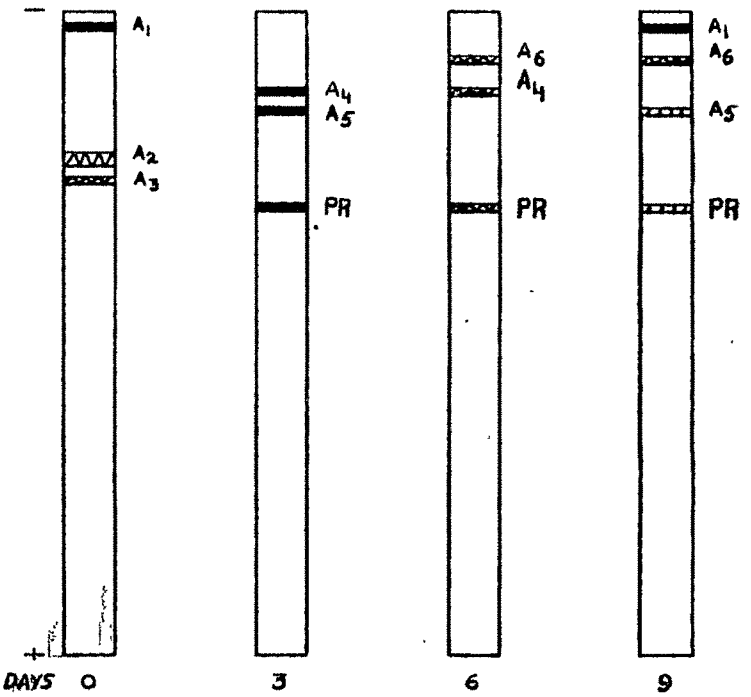


FIG. 100 a.

Anodic and Cathodic Isoperoxidase Patterns.
Experimental Details as in Table 38.



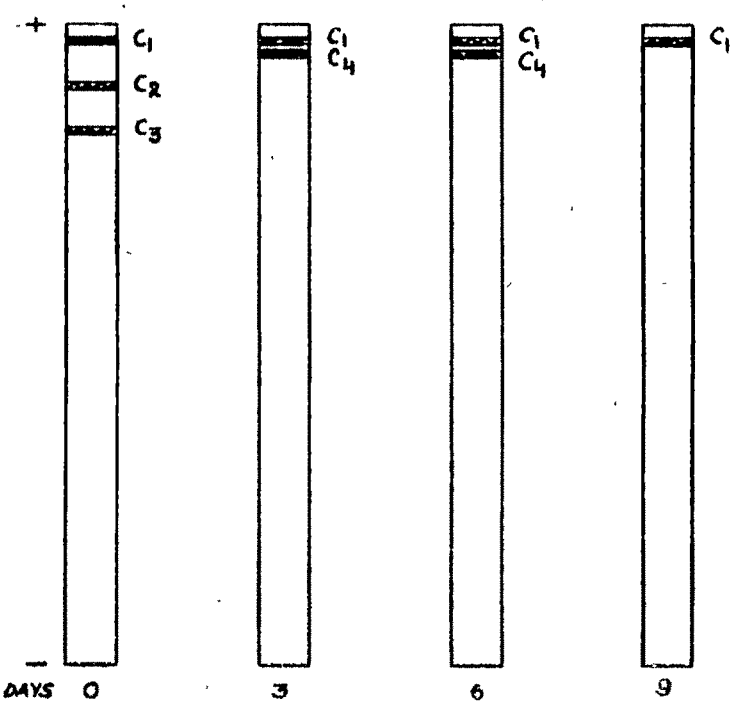
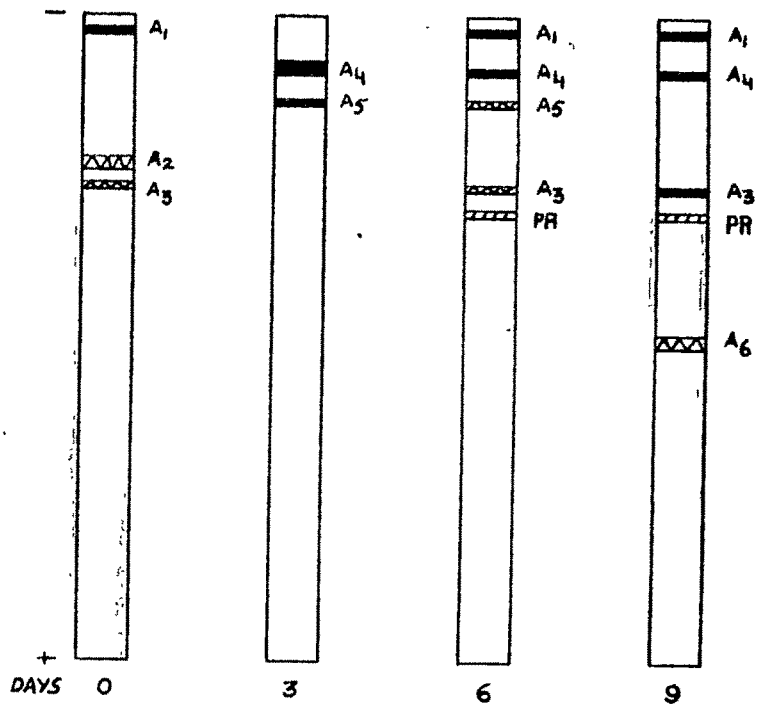


FIG. 101 a.

Anodic and Cathodic Isoperoxidase Patterns

Experimental Details as in Table 39.



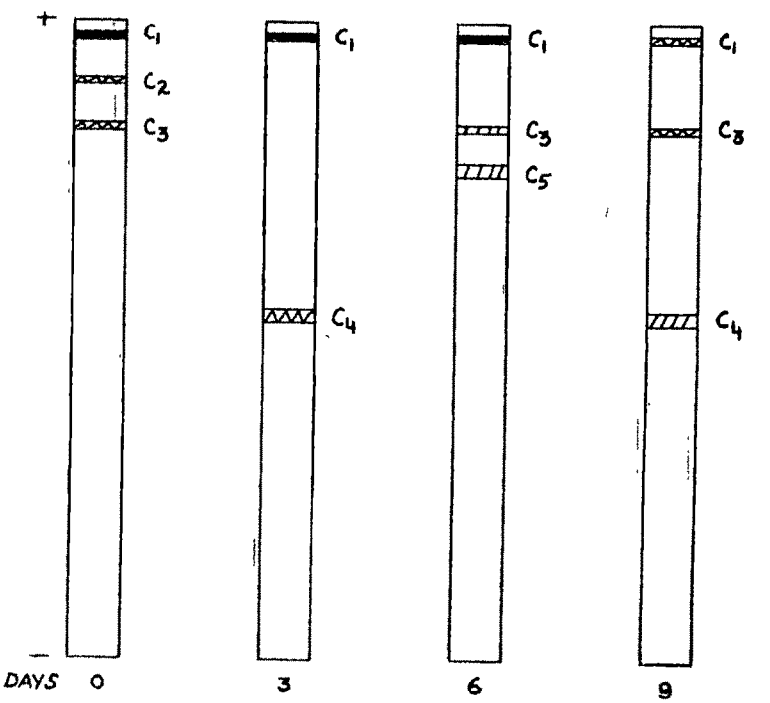
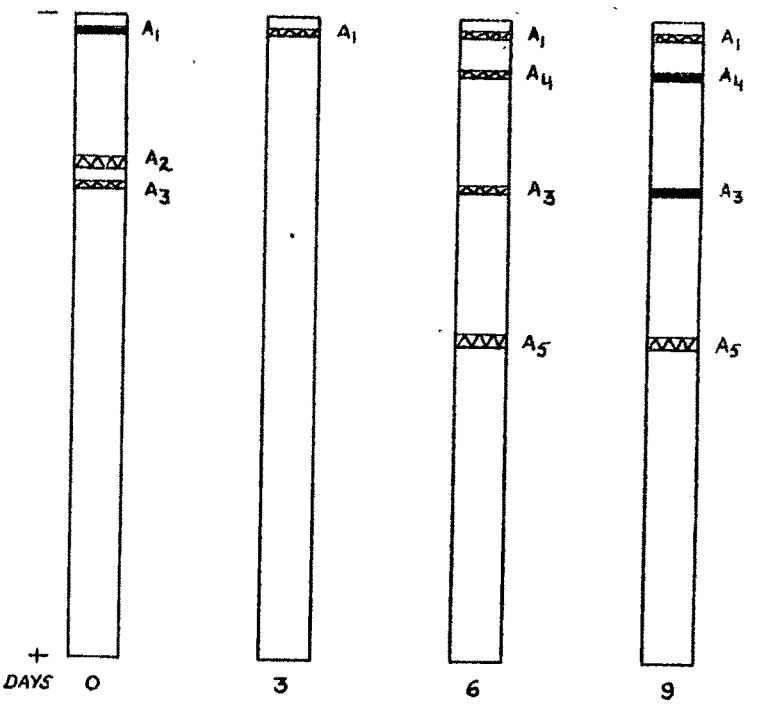


FIG. 102a

Anodic and Cathodic Isoperoxidase Patterns.

Experimental Details as in Table 40



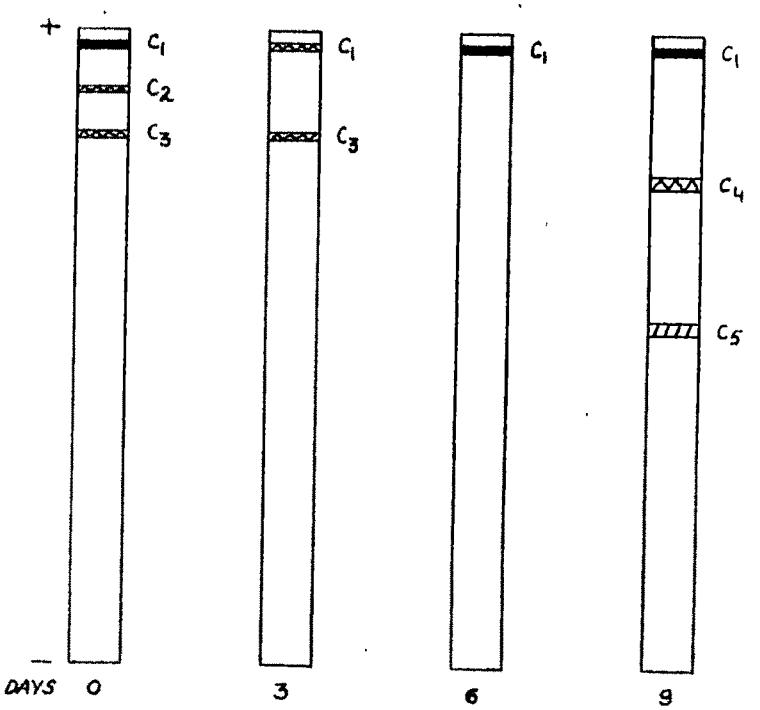
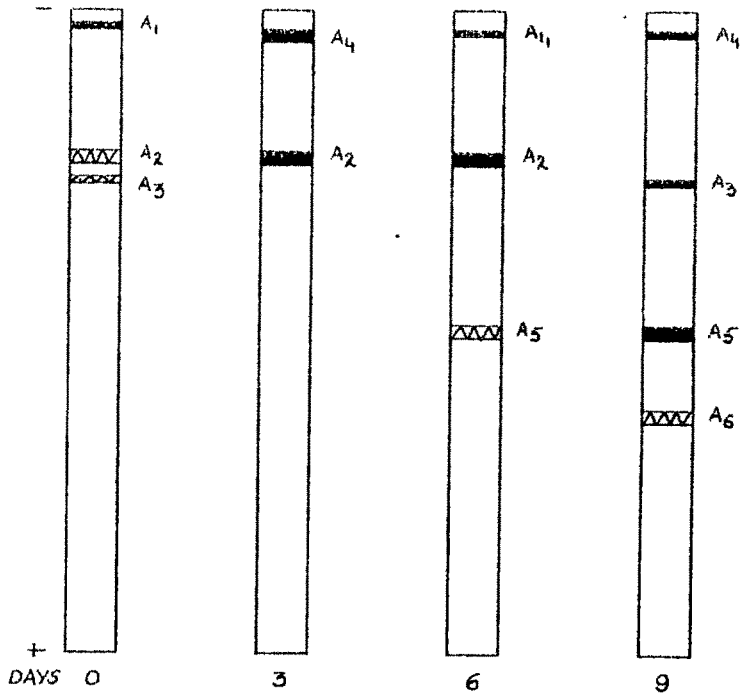


FIG. 103 a.

Anodic and Cathodic Isoperoxidase Patterns.

Experimental Details as in Table 41.



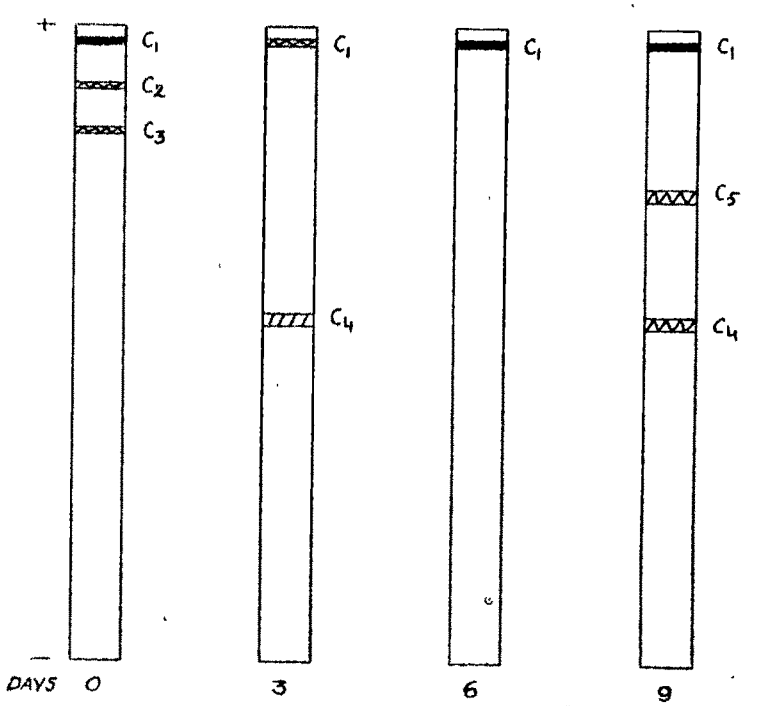


FIG. 104 a

Anodic and Cathodic Isoperoxidase Patterns.
Experimental Details as in Table 42.

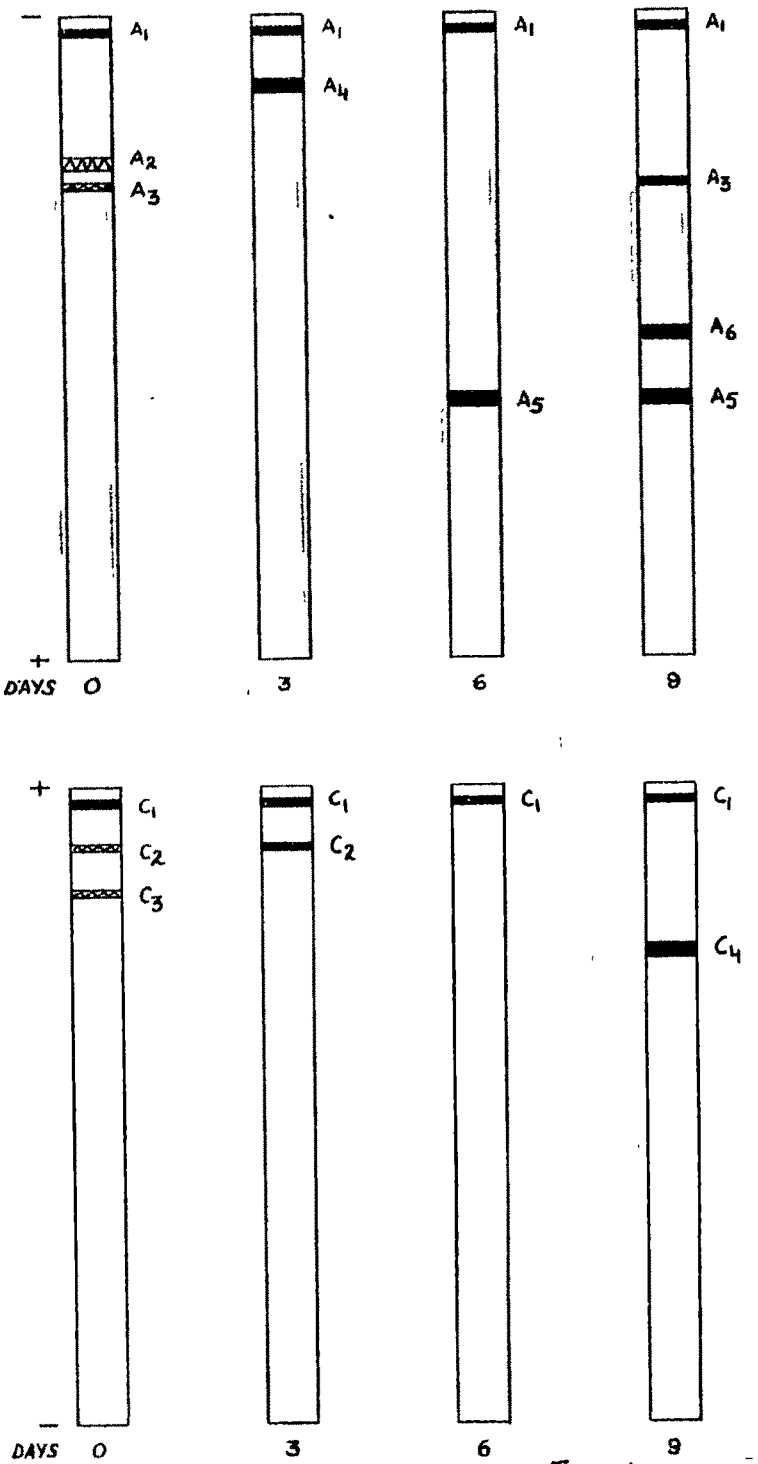


FIG. 105 a

Anodic and Cathodic Isoperoxidase Patterns
Experimental Details as in Table 43

Increase of caffeic acid to 500 µM in the root inducing medium resulted in differentiation of roots (on day 6) which was preceded by synthesis of anodic isoperoxidase "PR" on day 3, and continued to appear till day 9. The original anodic isoperoxidase A_1 was synthesized only on day 9. The other two initial isoperoxidases A_2 and A_3 were repressed throughout the culture period. Slow migrating anodic isoperoxidases A_4 on days 3 and 6; A_5 on days 3 and 9; and A_6 on days 6 and 9 were also synthesized. The two initial cathodic isoperoxidases C_2 and C_3 were not synthesized at all during the culture period. The initial cathodic isoperoxidase C_1, however, did appear on all days. A slow migrating isoperoxidase C_4 was synthesized on days 3 and 6 (Fig. 101a).

With incorporation of 100 µM ferulic acid into the root inducing medium, initial anodic isoperoxidase A_2, remained suppressed in haploid callus tissues throughout the culture period. Other two original anodic isoperoxidases A_1 and A_3 appeared on days 6 and 9. Two slow migrating anodic peroxidases A_4 on days 3, 6 and 9; and A_5 on days 3 and 6 were also synthesized. A_6, a fast migrating isoperoxidase was synthesized on day 9. Though the incorporation of 100 µM ferulic acid into root inducing medium suppressed differentiation of roots, it nevertheless, brought about the synthesis of anodic isoperoxidase "PR" which was associated with root differentiation

elsewhere. On same medium of the three initial cathodic isoperoxidases, C_2 remained suppressed throughout the culture period. C_1, however, appeared on all days and C_3 on days 6 and 9. A fast migrating cathodic isoperoxidase C_4 was synthesized on days 3 and 9. On day 6 a slow migrating one C_5, was also synthesized (Fig. 102a).

Increase of ferulic acid to 500 µM in root inducing medium suppressed differentiation of roots, as well as the synthesis of anodic isoperoxidase "PR". Of the three initial anodic isoperoxidases A_1 appeared on all days and A_3 on days 6 and 9. The third initial anodic isoperoxidase A_2 remained suppressed throughout the culture period. A slow migrating anodic isoperoxidase A_4 was synthesized on days 6 and 9. A fast migrating one A_5 was also synthesized on days 6 and 9. One initial cathodic isoperoxidase C_1, appeared on all days of culture; C_2 was suppressed for the entire length of culture period; and C_3 appeared only on day 3. A slow migrating cathodic isoperoxidase C_4 and a fast migrating one C_5, were synthesized on day 9 (Fig. 103a).

With the addition of 1.0 µM p-hydroxybenzoic acid in root inducing medium, the initial anodic isoperoxidase A_1 was suppressed for the entire length of the culture period. Second initial anodic isoperoxidase A_2 was synthesized on days 3 and 6 only. The third one, A_3 appeared only on day 9. During the

culture period one slow migrating anodic isoperoxidase A_4 was synthesized on days 3, 6 and 9. Two fast migrating ones, A_5 on days 6 and 9, and A_6 on day 9, were also synthesized. The root anodic peroxidase "PR" remained suppressed, as did the differentiation of roots. Of the three cathodic isoperoxidases, only C_1 appeared during the culture period. The other two, C_2 and C_3, were completely suppressed. A fast migrating cathodic isoperoxidase C_4 appeared on days 3 and 9. On day 9 a slow migrating one C_5 was also synthesized (Fig. 104a).

Elevation of p-hydroxybenzoic acid to 10.0 μM in the root inducing medium resulted in suppression of the initial anodic isoperoxidase A_2. A_1 appeared on all days, and A_3 on day 9. A slow migrating one A_4 was synthesized on day 3. Fast migrating anodic isoperoxidases, A_5 on days 6 and 9, and A_6 on day 9 were also synthesized. The root isoperoxidase "PR" was not synthesized, as the roots differentiation was inhibited. On the cathodic scale, the initial cathodic isoperoxidase C_3 remained suppressed for the entire culture period. Of the other two initial cathodic isoperoxidases, C_1 appeared on all days and C_2 on day 3 only. On day 9 a slow migrating isoperoxidase C_4 was also synthesized (Fig. 105a).

Expt. 33. Studies with haploid callus tissues cultured on root inducing medium containing ½ x and 2 x Mn^{++} ion concentrations.

Healthy callus masses of N. tabacum of haploid origin, weighing 300±30 mg by fresh weight were cultured on 20 ml of the root inducing medium i.e., MS basal + 2.0 mg/l IAA + 3% sucrose. Here the MS basal stocks had been so adjusted as to contain only ½ x or 2 x normal level of Mn^{++} ions. Ordinarily MS basal medium contains 22.3 mg/l of $MnSO_4$. In the present experiment MS basal medium was prepared with 11.15 mg/l (½ x normal) and 44.6 mg/l (2 x normal) $MnSO_4$.

The culture vessels were incubated at 26±2° in continuous light. Every three days till day 15, a fixed number of 5 replicates was harvested and analysed for growth, enzymes, peroxidase isoenzymes and phenolics.

(a) Growth :

The growth of haploid tobacco tissues cultured on IAA containing root inducing medium, in which ½ x and 2 x Mn^{++} ion concentrations were used, is illustrated in Figs. 106, 107, and Tables 44, 45.

The growth of haploid callus was inhibited as compared with control, when in the root inducing medium the Mn^{++} level was either reduced to ½ of the normal level or elevated to

double its concentration. On medium containing $\frac{1}{2}$ x Mn^{++} ion level the respective fresh and dry weight increases were 1.81 and 3.53 fold (Fig. 106, Table 44). On medium containing 2 x Mn^{++} ions the fresh and dry weight increases were 1.78 and 3.51 fold (Fig. 107, Table 45). On the other hand, the fresh and dry weight increases on the control medium were 2.84 and 5.6 fold respectively.

With the use of $\frac{1}{2}$ x and 2 x level of Mn^{++} ion in the IAA (2.0 mg/l) containing root differentiating medium, the differentiation of roots from haploid tobacco callus tissues was inhibited. On the control medium the same organogenetic response was obtained on day 6 of culture.

(b) Peroxidase :

The progressive changes in the total and specific peroxidase activity of haploid tobacco callus cultured on IAA (2.0 mg/l) containing medium in which $\frac{1}{2}$ x and 2 x Mn^{++} ion were used, are illustrated in Figs. 106, 107 and Tables 44, 45.

On suboptimal Mn^{++} ion containing medium the total peroxidase activity demonstrated double peaked pattern of development. The first peak was reached on day 6 and the second on day 15. On the other hand, the specific peroxidase activity declined slightly during the initial three days of

culture. Thereafter the activity was on the increase right upto day 15 (Fig. 106, Table 44).

With the use of supraoptimal Mn^{++} ion level in the medium the total and specific peroxidase activities demonstrated identical developmental pattern, which was a double peaked one. The peroxidase activity during the initial three days of culture declined slightly. Thereafter, it increased steadily to the first peak on day 9. By day 12 the activity had declined sharply, but made a slight come back by day 15 (Fig. 107, Table 45).

The total peroxidase activity per unit protein of haploid callus cultured on standard root inducing medium as well as on the root inducing medium containing $\frac{1}{2}$ x and 2 x Mn^{++} ion levels, was on the decline during the first three days of culture. On the control medium the peroxidase activity increased between days 3-6 i.e., before root differentiation on day 6 and declined thereof with root differentiation. On $\frac{1}{2}$ x Mn^{++} ion containing medium the enzyme activity was continuously on the increase between days 3 and 15. On 2 x Mn^{++} ion containing medium the activity increased between days 3 and 9, declined between 9 and 12 days and then increased again till day 15. On the root inducing medium containing $\frac{1}{2}$ x and 2 x Mn^{++} ion levels root differentiation was totally inhibited.

Table : 44. Growth, Polyphenols and progressive changes in the Activity of Peroxidase, IAA Oxidase, MDH and PAL in haploid callus tissues of N. tabacum L. var. Anand-2 cultured on root differentiating medium containing ½ X Mn^{++} ion concentration.

Medium : MS(containing ½ X Mn^{++} ion conc.) + 2.0 mg/l IAA + 3% sucrose

Incubation : 15 days at 26±2° in continuous light

Inoculum : 300±30 mg fresh tissue

Day	Fresh weight mg/cult.	Dry weight mg/cult.	PEROXIDASE		IAA OXIDASE		MDH		PAL		PHENOLICS	
			units/ cult.	units/mg protein	units/ cult.	units/mg protein	units/ cult.	units/mg protein	units/ cult.	units/mg protein	mg/ cult.	mg%
0	300 (±30)	12 (±3)	0.98	0.86	0.48	0.42	1.05	0.92	0.28	0.25	0.13	1.12
3	388.5 (±21.8)	23.5 (±2.3)	1.11	0.78	4.94	3.48	1.86	1.31	0.86	0.60	0.31	1.32
6	499.2 (±39.2)	35.9 (±3.3)	1.67	1.16	1.56	1.08	4.79	3.32	16.08	11.16	0.86	2.42
9	512.3 (±42.5)	36.8 (±3.8)	1.36	1.38	10.11	10.22	4.18	4.23	15.53	15.69	1.00	2.89
12	526.0 (±38.5)	39.3 (±1.9)	0.90	1.45	14.09	22.72	5.78	9.33	11.68	18.85	1.56	3.98
15	543.5 (±44.4)	42.4 (±2.6)	1.68	1.56	13.83	12.81	10.87	10.06	18.11	16.77	1.59	3.75

Figures in parenthesis represent standard error.

Data represented is average of 5 replicates.

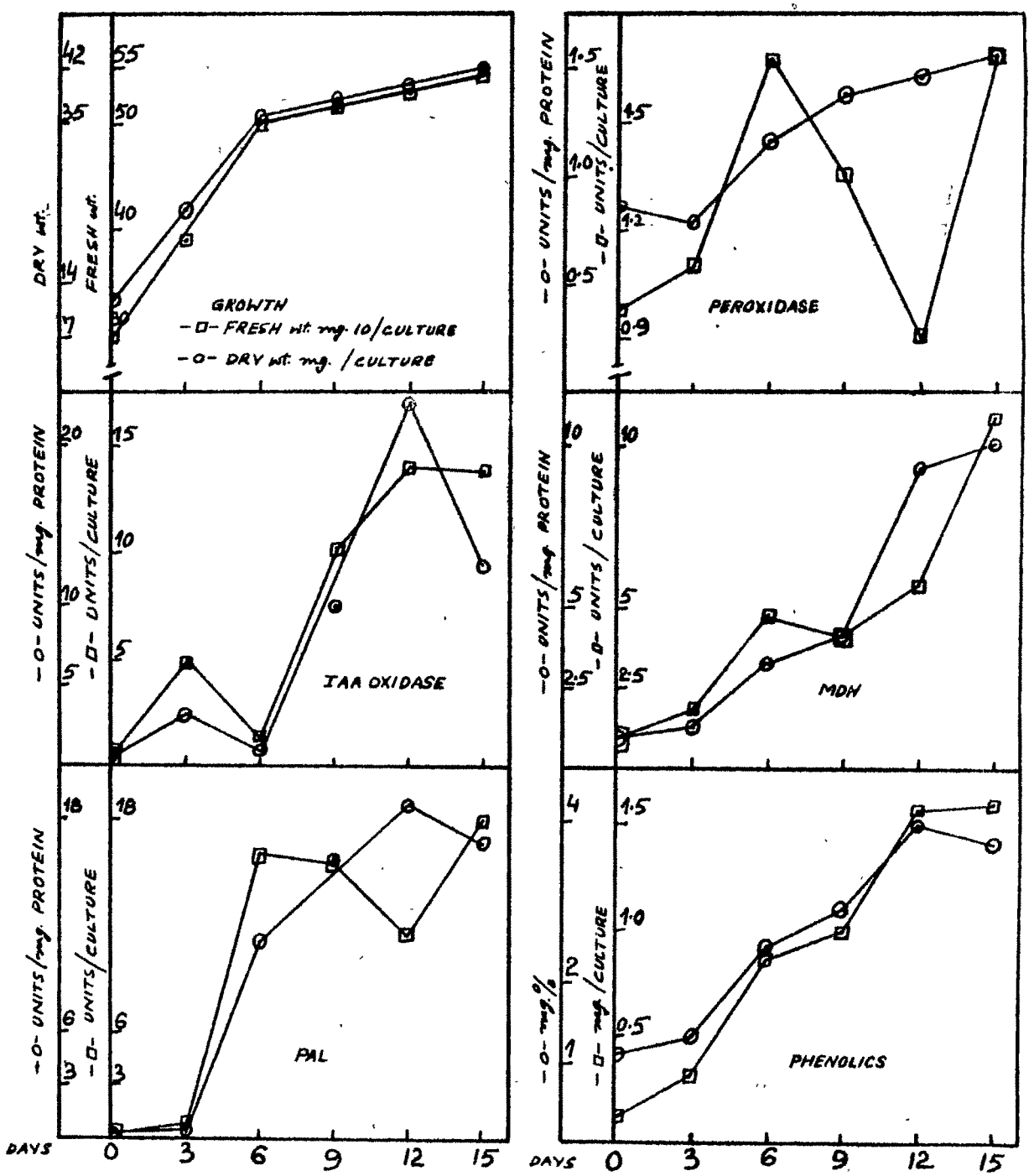


FIG. 106.

Experimental Details as in Table 44.

Table : 45. Growth, Polyphenols and progressive changes in the Activity of Peroxidase, IAA Oxidase, MDH and PAL in haploid callus tissues of N. tabacum L. var. Anand-2 cultured on root differentiating medium containing 2 X Mn^{++} ion concentration.

Medium : MS (containing 2 X Mn^{++} ion conc.) + 2.0 mg/l IAA + 3% sucrose

Incubation : 15 days at 26±2° in continuous light

Inoculum : 300±30 mg fresh tissue

Day	Fresh weight mg/cult.	Dry weight mg/cult.	PEROXIDASE		IAA OXIDASE		MDH		PAL		PHENOLICS	
			units/ cult.	units/mg protein	units/ cult.	units/mg protein	units/ cult.	units/mg protein	units/ cult.	units/mg protein	mg/ cult.	mg%
0	300 (±30)	12 (±3)	0.98	0.86	0.48	0.42	1.05	0.92	0.28	0.25	0.13	1.12
3	402.1 (±28.9)	24.9 (±2.9)	0.61	0.81	2.85	3.80	0.72	0.96	2.01	2.68	0.46	1.87
6	504.3 (±30.4)	37.8 (±3.6)	2.56	1.56	2.57	1.56	3.63	2.21	19.04	11.61	0.99	2.63
9	521.6 (±41.7)	39.4 (±3.8)	3.13	2.21	20.70	14.58	13.75	9.69	19.56	13.78	1.23	3.12
12	534.0 (±32.6)	42.0 (±2.4)	0.91	1.05	15.10	17.36	12.81	14.73	4.74	5.45	0.84	2.02
15	535.0 (±32.9)	42.2 (±1.8)	1.59	1.21	12.94	9.88	10.27	7.84	11.88	9.07	0.80	1.89

Figures in parenthesis represent standard error.

Data represented is average of 5 replicates.

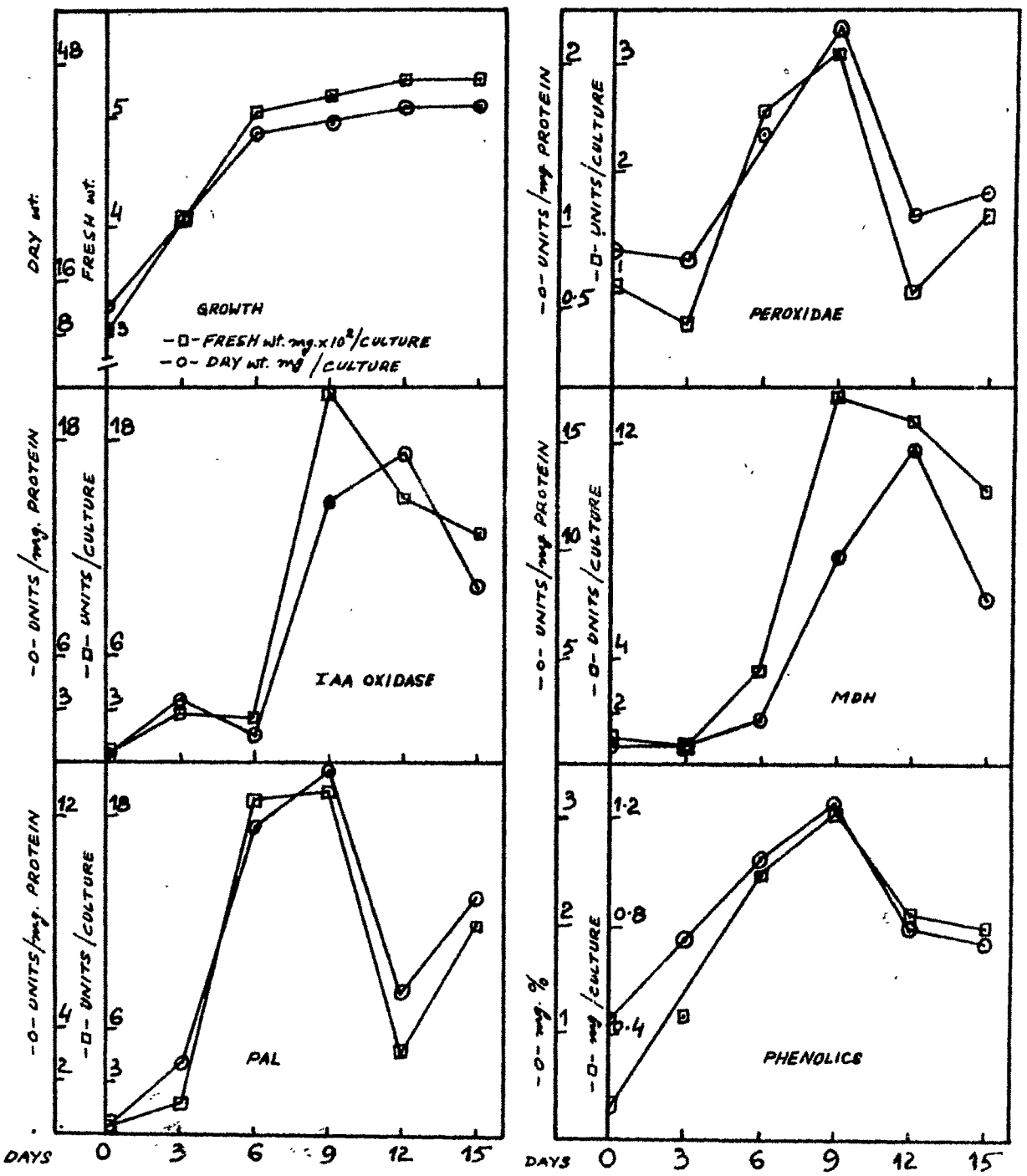


FIG. 107

Experimental Details as in Table 45.

(c) IAA Oxidase :

The progressive changes in the total and specific activities of IAA Oxidase in haploid tobacco callus tissues cultured on IAA (2.0 mg/l) containing medium in which sub- and supra- optimal concentrations of Mn^{++} were used, are illustrated in Figs. 106, 107 and Tables 44, 45.

The total and specific activities of IAA Oxidase in haploid tobacco callus tissues on suboptimal Mn^{++} ion containing medium, demonstrated identical double peaked developmental pattern. The first peak of total and specific IAA Oxidase activity was reached on day 3. By day 6 the activity declined. Thereafter till day 12 the activity was on the increase and the second peak reached. However, by day 15 the activity declined again (Fig. 106, Table 44).

On supraoptimal Mn^{++} ion containing medium the total IAA Oxidase activity in haploid tissues increased during the first three days of culture. Between days 3 and 6 the activity declined, but only marginally. The peak value was reached on day 9 as a result of a very sharp increase in the activity. However, between days 9 and 15 the activity was continuously on the decline. On the other hand, the specific activity of IAA Oxidase on the same medium demonstrated double peaked pattern of development. The first peak value was attained on day 3 and the second on day 12. Between days 3 and 6, and 12

and 15 the activity declined (Fig. 107, Table 45).

The total IAA Oxidase activity per unit protein of haploid callus on standard rooting medium was on the increase during the days preceding root differentiation (day 3-6) and on decline thereafter (day 6-9) with the use of $\frac{1}{2}$ x and 2 x Mn^{++} ion levels in the same rooting medium the enzyme activity exhibited double peaked pattern of development. The first peak value was reached on day 3 and the second on day 15. Between day 3 and 6 the enzyme activity was on the decline and then on increase between days 6-9.

(d) MDH :

The Figs. 106, 107 and Tables 44, 45, illustrate progressive changes in the total and specific activities of MDH in haploid tobacco callus cultured on IAA (2.0 mg/l) containing medium in which sub- and supra-optimal levels of Mn^{++} ion were used.

The total MDH activity in haploid tobacco callus tissues cultured on medium containing sub-optimal level ($\frac{1}{2}$ x) Mn^{++} ions, demonstrated double peaked developmental pattern. During the first 6 days of culture the activity was on the increase and attained its first peak value. The activity declined, however, between days 6 and 9. Thereafter, the activity increased unabated till day 15 reaching its second

peak value. On the contrary, the specific activity of MDH demonstrated incessant increase during the entire culture period of 15 days (Fig. 106, Table 44).

On supra-optimum (2 x) Mn^{++} ion containing medium the total MDH activity demonstrated a slight decline during the initial 3 days of culture. Thereafter, it increased sharply till day 9 to reach its peak value. The activity, however, declined between days 9 and 15. The specific activity of MDH, on the other hand, exhibited a very modest increase in activity between days 0 and 3. Thereof the activity increased unabated till day 12 attaining its peak value. The activity, however, declined thereafter till day 15 (Fig. 107, Table 45).

The total MDH activity per unit protein of haploid callus tissues cultured on standard root inducing medium increased sharply during the days preceding root differentiation (days 3-6). Following root differentiation the enzyme activity declined (days 6-9). With the incorporation of $\frac{1}{2}$ x and 2 x Mn^{++} ion levels in the root inducing medium the MDH activity of haploid callus during the period of days 3-6 increased but rather marginally in comparison with the increase on standard medium. The activity on $\frac{1}{2}$ x Mn^{++} ion containing medium continued to increase till day 15, whereas on 2 x Mn^{++} containing medium the activity increased till day 12 and declined thereafter.

(e) PAL :

Figs. 106, 107 and Tables 44, 45 illustrate the progressive changes in the total and specific activities of PAL in haploid tobacco callus tissues cultured on IAA (2.0 mg/l) containing medium in which sub- and supra-optimal ($\frac{1}{2}$ x and 2 x) Mn^{++} ion levels were used.

In haploid tobacco callus tissues cultured on sub-optimal ($\frac{1}{2}$ x) Mn^{++} ion containing medium the total PAL activity demonstrated double peaked developmental pattern. During the initial 3 days of culture, the activity demonstrated only marginal increase. The activity, however, shot up thereof and attained its first peak value on day 6. Between days 6 and 12 the PAL activity was on the decline. By day 15 it again made a come back and attained its second peak value on day 15. On the other hand, the specific PAL activity was on the increase between days 0 and 12, whence the peak value was attained. Thereafter, the activity declined till day 15 (Fig. 106, Table 44).

The total PAL activity in haploid tobacco callus tissues cultured on supra-optimal (2 x) Mn^{++} ion containing medium increased sharply between days 0 and 6. Between days 6 and 9, the activity increased only marginally and the first peak value was reached on day 9. A sharp decline in the activity was registered between days 9 and 12, but thereafter, it

climbed up again to its second peak value by day 15. The specific PAL activity also demonstrated similar developmental pattern. The PAL activity increased unabated during the first 9 days of culture. A sharp decline in activity ensued thereafter, till day 12. Between days 12 and 15 the activity increased again to its e second peak value (Fig. 107, Table 45).

The total PAL activity per unit protein of haploid callus tissue cultured on standard root inducing medium increased between days 3-6 i.e., the days immediately preceding root differentiation, and declined between days 6-9, i.e., the days immediately after root differentiation. With the use of $\frac{1}{2}$ x and 2 x Mn^{++} ion levels in the root inducing medium though the enzyme activity was on the increase between days 3-6, the value reached on day 6 was about 12 times more than on the standard rooting medium. Furthermore, during the days 6-9 the enzyme activity instead of declining, as was the case on standard rooting medium, continued to increase.

(f) Phenolics :

Figs. 106, 107 and Tables 44, 45 present progressive changes in the accumulation of phenolics in haploid tobacco callus tissues cultured on IAA (2.0 mg/l) containing medium in which sub- and supra- optimal ($\frac{1}{2}$ x and 2 x) Mn^{++} ion levels were used.

On sub-optimal Mn^{++} ion containing medium the phenolics accumulated in haploid tobacco callus throughout the 15 days of culture period. It increased from 0.13 mg/culture on day 0 to 1.59 mg/culture on day 15. On percentage basis, the peak phenolic accumulation was reached on day 12. It increased from 1.12 mg% on day 0 to 3.98 mg% on day 12. By day 15 it had, however, declined slightly to 3.75 mg% (Fig. 106, Table 44).

In haploid tobacco callus tissues cultured on supra-optimal Mn^{++} ion containing medium the phenolics increased from 0.13 mg/culture on day 0 to 1.23 mg/culture on day 9. Between days 9 and 15 it was on decline, and reached the value of 0.80 mg/culture on day 15. On percentage basis, also the peak phenolic accumulation was attained on day 9. It increased from 1.12 mg% on day 0 to 3.12 mg% on day 9. Thereafter till day 15 it declined continuously and reached the value of 1.89 mg% on day 15 (Fig. 107, Table 45).

The phenolic content of haploid tobacco callus cultured on root inducing medium increased from 0.96 mg% to 1.37 mg% during days immediately preceding root differentiation (days 3-6) and to 2.38 mg% immediately after it between days 6-9. During the corresponding periods the phenolic content on $\frac{1}{2}$ x Mn^{++} ion containing medium increased from 0.31 mg% to 0.86 mg% (between days 3-6) and finally to 1.0 mg% (on

day 9). On the use of 2 x Mn^{++} ion in the medium the phenolic content increased from 0.46 mg% to 0.99 mg% (between days 3-6) and then to 1.23 mg% (on day 9). Hence, the phenolic accumulation in the root forming calli was more than in non-root forming ones.

(g) Peroxidase Isoenzymes :

Illustrated in Figs. 106a, 107a are the progressive changes of anodic and cathodic isoperoxidases of haploid tobacco callus tissues cultured on IAA (2.0 mg/l) containing medium in which sub- and supra- optimal levels ($\frac{1}{2}$ x and 2 x) of Mn^{++} ions were used.

On sub- optimum Mn^{++} containing medium the original anodic isoperoxidases A_1 and A_3 remained suppressed right through the culture period. The third one A_2, however, was synthesized between days 9-15. During the culture period five slow migrating anodic isoperoxidases A_4-A_8,were synthesized. A_4 and A_5 appeared on all days, A_6 on days 3, 12, and 15, A_7 on day 3 and A_8 between days 6-15. On the cathodic scale the original cathodic isoperoxidase C_3 was not synthesized at any time during the 15 day culture period. The other original cathodic isoperoxidase C_1 appeared on all days and C_2 was synthesized only on day 15. Three slow migrating ones were also synthesized during the culture

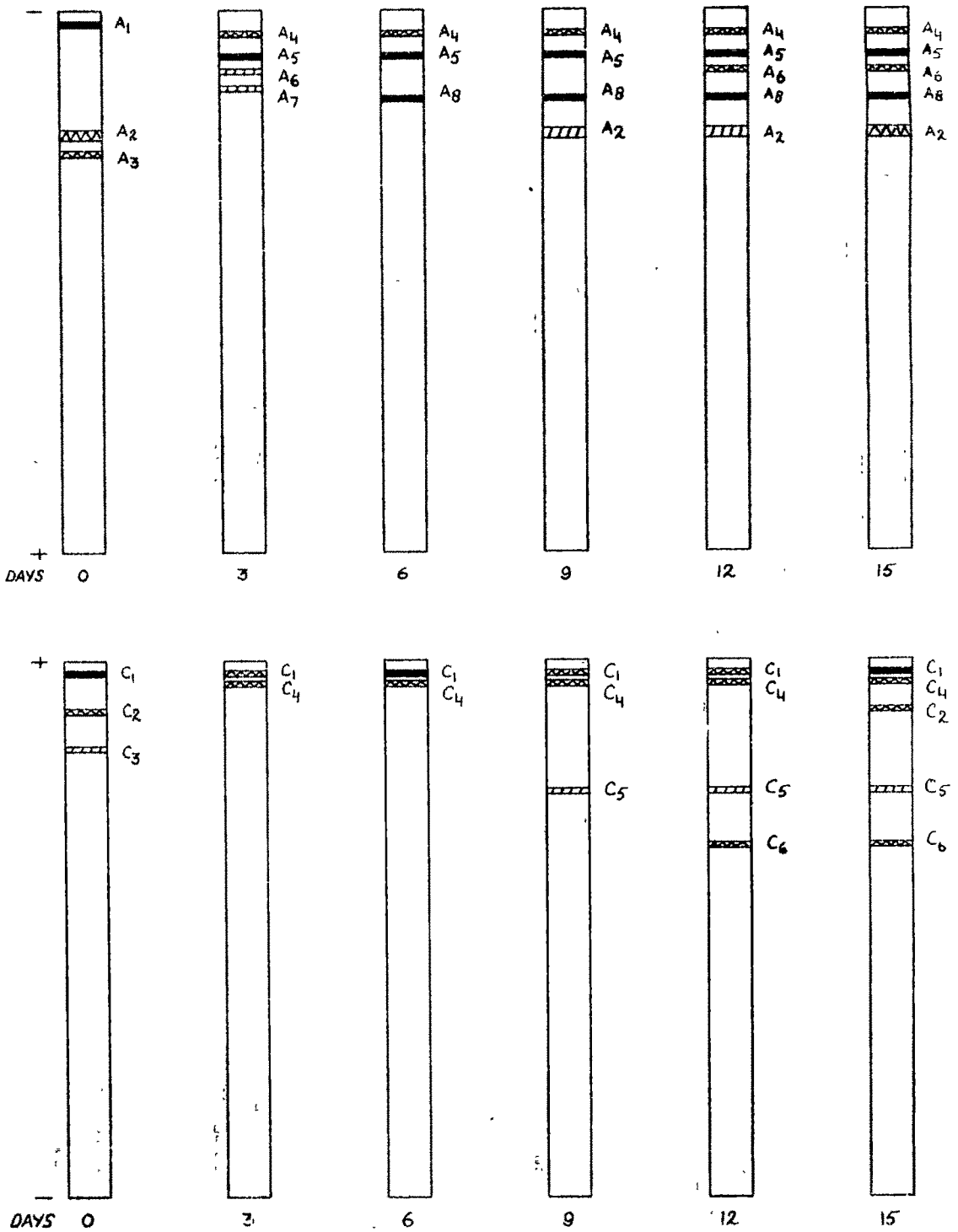


FIG. 106a.

Anodic and Cathodic Isoperoxidase Patterns.

Experimental Details as in Table 44.

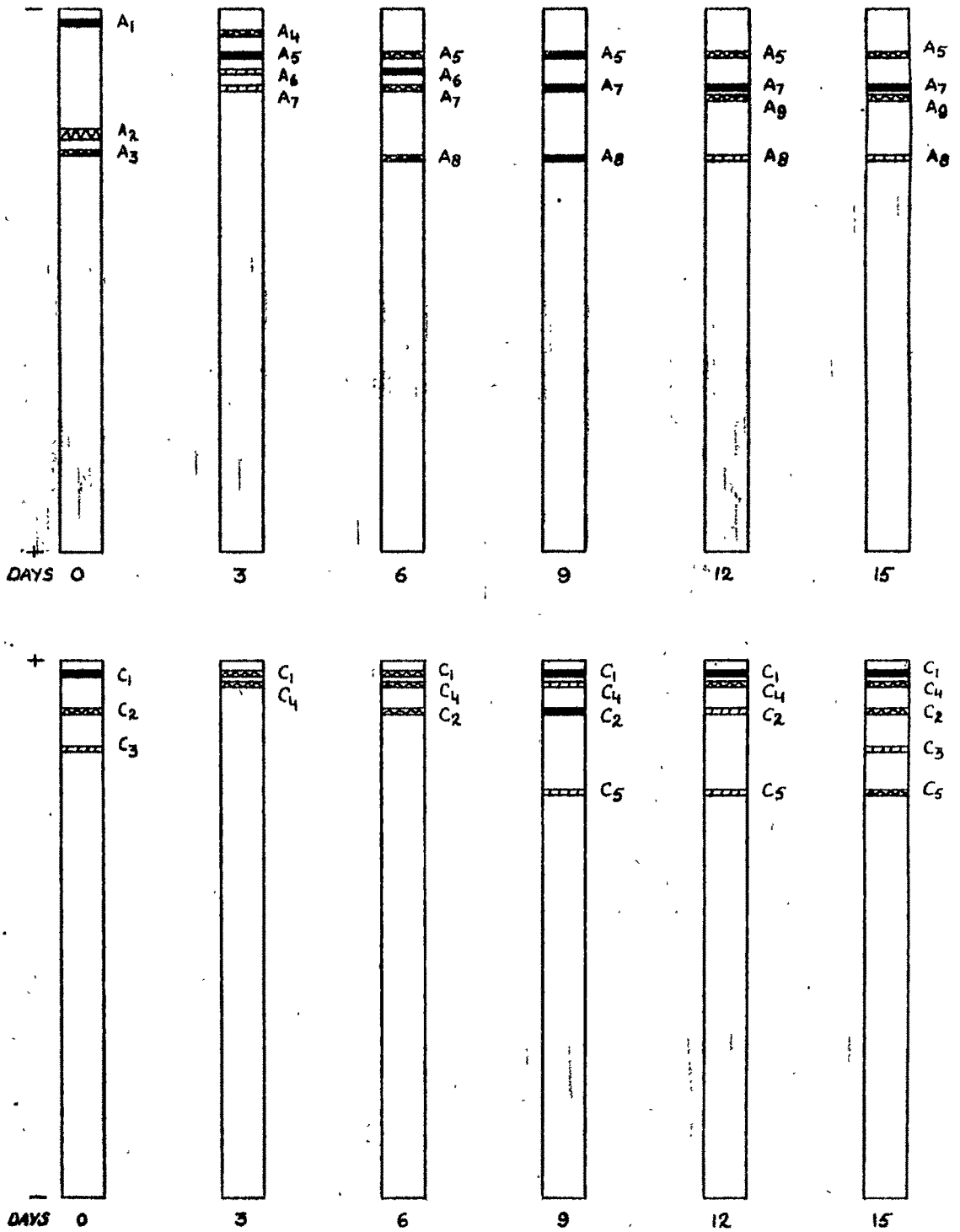


FIG. 107a

Anodic and Cathodic Isoperoxidase Patterns.
Experimental Details as in Table 45.

period. Of these C_4 appeared on all days, C_5 on days 9, 12 and 15, and C_6 on days 12 and 15 (Fig. 106a).

All the three original anodic isoperoxidases A_1, A_2 and A_3 remained suppressed in haploid tobacco callus tissues when cultured on supra- optimum Mn^{++} ion containing medium. During the culture period six slow migrating anodic isoperoxidases A_4-A_9, were synthesized. A_4 appeared on day 3, A_5 and A_7 were synthesized on all days, A_6 on days 3 and 6, A_8 from day 6 onwards till day 15 and A_9 on days 12 and 15. On the cathodic scale the original cathodic isoperoxidase C_1 was synthesized on all days of culture. C_2, other original cathodic isoperoxidase, remained suppressed only on day 3. The third original one C_3 was synthesized only on day 15. During the culture period two slow migrating cathodic isoperoxidases C_4 and C_5 were synthesized. C_4 appeared on all days of culture and C_5 on days 9, 12 and 15 (Fig. 107a).

On this IAA (2.0 mg/l) containing root inducing medium the alteration of Mn^{++} level to either $\frac{1}{2}$ x or 2 x of the normal level, reflected in suppression of root differentiation. Furthermore, the synthesis of root peroxidase "PR" was also inhibited.

Section F : Physiological Studies with Peroxidase, IAA Oxidase, MDH, PAL, Peroxidase Isoenzymes and Phenolics in Diploid and Haploid callus tissues of *N. tabacum* cultured on shoot differentiating medium.

From the experiments carried out earlier and described in Sections C-I and C-II of Chapter III (Results), it became obvious that diploid and haploid callus cultures of N. tabacum retained fully their morphogenetic potentials in vitro. It was the exogenous supply of growth hormones, singly or in combinations, which determined the organogenetic responses of these callus cultures.

To examine physiological changes associated with shoot differentiation in diploid and haploid tobacco callus tissues, the parameters studied were : (i) Growth, (ii) Peroxidase, (iii) IAA Oxidase, (iv) MDH, (v) PAL, (vi) Peroxidase isoenzyme patterns and (vii) Phenolic accumulation.

The shoot differentiating medium used was : MS (basal) + 0.3 mg/l IAA + 3% sucrose. Shoots were differentiated from callus tissues of tobacco on the above medium in a maximum of 15 days from diploid callus and within 9 days from haploid callus tissues with around 75 per cent frequency. The experiments were terminated as soon as morphogenic responses were manifested or else were carried on till day 15 of culture. During this period, commencing with day 0, callus tissues were harvested every third day and analysed for the parameters enlisted above.

The level of sucrose in culture medium is reported to

influence morphogenetic response of tissue cultures (Chapter I, Introduction). In the present study also, it became clear that alteration of sucrose level in medium had profound influence on the degree of response and time taken for its expression. Also it was amply demonstrated that with alteration of sucrose level in the medium, a shift occurred in the morphogenetic response from rhizogenesis to caulogenesis or vice versa. (Chapter III, Results, Sections C-I and C-II).

Earlier studies mentioned in Chapter I (Introduction) indicated that hydroxylated aromatic compounds like phenolic acids influenced organogenesis in cultured tissues. Effectiveness of these phenolic compounds was correlated with enhanced peroxidase IAA Oxidation. It was also proposed that certain phenolics stimulate IAA inactivation, thus creating a situation favourable for organogenesis. Some phenolics are also acredited with the function of being auxin protectors, thereby preventing its destruction. In the present study (Chapter III, Results; Sections C-I and C-II) it was observed that addition of certain phenolic acids into the shoot inducing medium, either brought about complete inhibition of caulogenesis or created a situation favouring root differentiation.

The requirement of Mn^{++} ions for growth and its continuation has been amply demonstrated by earlier studies.

Its requirement for IAA Oxidation and also its influence on PAL have also been greatly elaborated (Chapter I, Introduction). On account of varied physiological roles attributed to Mn^{++}, effects of different doses of Mn^{++} on shoot differentiation and associated physiological parameters were looked into.

For the sake of convenience the results in this section are presented under the following heads :

(i) Studies with floral bud callus cultured on shoot inducing medium,

(ii) Studies with floral bud callus cultured on shoot inducing medium supplemented with low and high levels of sucrose,

(iii) Studies with floral bud callus cultured on shoot inducing medium supplemented with various phenolic acids,

(iv) Studies with floral bud callus cultured on shoot inducing medium containing $\frac{1}{2}$ x and 2 x Mn^{++} ion concentrations,

(v) Studies with haploid callus tissues cultured on shoot inducing medium,

(vi) Studies with haploid callus tissues cultured on shoot inducing medium supplemented with low and high levels of sucrose,

(vii) Studies with haploid callus tissues cultured on shoot indicing medium supplemented with various phenolic acids,

(viii) Studies with haploid callus tissues cultured on shoot inducing medium containing $\frac{1}{2}$ x and 2 x Mn^{++} ion concentrations.

Expt. 34. Studies with floral bud callus cultured on shoot inducing medium.

Healthy callus masses weighing 300±30 mg by fresh weight were selected and cultured on 20 ml MS basal medium supplemented with 0.3 mg/l IAA and 3% sucrose. The culture vessels were incubated at 26±2° in continuous light for a period of 15 days. Every third day, 5 replicates were harvested and analysed for growth, enzymes, isoperoxidase patterns and phenolic content. The results are described below.

(a) Growth :

Growth of floral bud callus cultured on shoot inducing medium and measured as increase in fresh and dry weight is illustrated in Fig. 108 and Table 46.

Growth of floral bud callus, cultured on shoot inducing medium, on fresh and dry weight basis increased rather rapidly between days 0 and 3. Between days 3 and 6, the growth rate was comparatively slower. The rate was again fast between days 6 and 9. Between days 9 and 15 the growth was on increase but at a very slow rate. During days 0 to 15, fresh weight increased 1.64 fold and dry weight 3.63 fold.

On the above medium differentiation of shoots occurred from floral bud callus on day 15 of culture with over 75 per cent frequency.

Table :46 Growth, Polyphenols and progressive changes in the Activity of Peroxidase, IAA oxidase, MDH and PAL during shoot differentiation from Diploid callus tissues of N. tabacum L. var. Anand-2.

Medium : MS + 0.3 mg/l IAA + 3% sucrose

Incubation : 15 days at 26±2° in continuous light

Inoculum : 300±30 mg fresh weight

Day	Fresh weight mg/cult.	Dry weight mg/cult.	PEROXIDASE		IAA OXIDASE		MDH		PAL		PHENOLICS	
			units/ cult.	units/mg protein	units/ cult.	units/mg protein	units/ cult.	units/mg protein	units/ cult.	units/mg protein	mg/ cult.	mg%
0	300 (±30)	12 (±3)	1.77	0.92	0.57	0.30	1.69	0.88	0.48	0.25	0.22	1.87
3	382.0 (±33)	18.6 (±2.3)	0.59	0.55	1.68	1.57	3.57	3.32	0.04	0.04	0.61	3.30
6	392.6 (±24)	21.2 (±3.5)	0.99	0.82	4.34	3.51	2.88	2.33	0.06	0.05	0.92	3.30
9	465.0 (±41)	35.0 (±4.7)	1.88	1.07	7.54	4.29	1.74	0.99	2.68	1.53	1.06	3.05
12	479.3 (±21.9)	40.3 (±8.9)	1.54	0.66	9.31	3.99	3.12	1.34	0.84	0.36	1.12	2.80
15	493.2 (±22.5)	43.6 (±6)	2.72	1.01	6.69	2.48	4.47	1.66	1.18	0.44	1.00	2.30

Figures in parenthesis indicate standard error.

Data represented is average of 5 replicates.

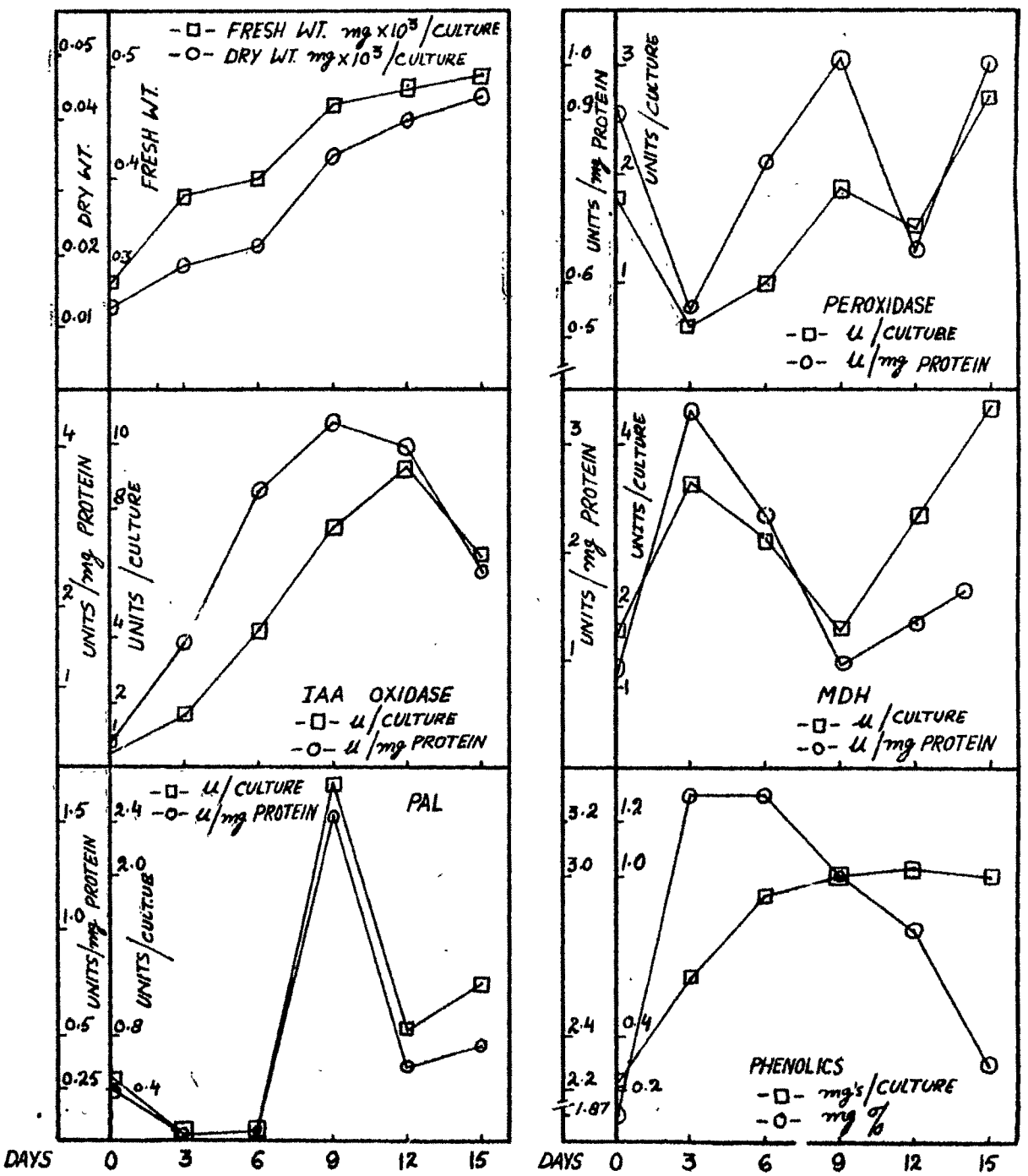


FIG. 108.

Experimental Details as in Table 46.

(b) Peroxidase :

Progressive changes of peroxidase activity in floral bud callus cultured on shoot inducing medium are illustrated in Fig. 108 and Table 46.

The peroxidase activity per culture and also per unit protein was on decline between day 0 and 3. Thereof the activity increased to reach its first peak value on day 9. Between days 9 and 12 the activity was again on the decline, but thereof till day 15 the activity increased. In other words, the peroxidase activity per culture was on the increase in the days (12-15) immediately preceding shoot differentiation.

(c) IAA Oxidase :

Changes in IAA Oxidase activity of floral bud callus cultured on shoot inducing medium are illustrated in Fig. 108 and Table 46.

The IAA oxidase activity per culture and per unit protein followed almost identical pattern of development. The activity per culture increased between days 0 and 12, and thereof declined till day 15. The peak value was reached on day 12. The activity per unit protein increased almost linearly between day 0 and 9, thereof till day 15 it was on decline. The IAA Oxidase activity of floral bud callus was

on the decline during the days preceding shoot differentiation on day 15.

(d) MDH :

The activity of MDH in floral bud callus cultured on shoot differentiating medium is illustrated in Fig. 108 and Table 46.

The MDH activity per culture and per unit protein follow identical patterns of development. during the 15 day culture period. The activity on both the accounts increased rather rapidly between days 0 and 3. However, it was on the decline from day 3 to 9. The activity increased linearly between days 9 and 15. So during the entire culture period two peak values were reached, one each on day 3 and 15.

The MDH activity on both accounts was on increase during the days immediately preceding (days 9 to 15) shoot differentiation.

(e) PAL :

Fig. 108 and Table 46 represent progressive changes of PAL activity per culture and per unit protein in floral bud callus cultured on shoot inducing medium.

The PAL activity per culture and per unit protein

exhibited similar patterns of development. The PAL activity on both accounts declined between days 0 and 3. It, however, remained more or less stable between days 3 and 6. Thereof PAL activity increased very rapidly to reach its peak value on day 9. Between days 9 and 12 it decayed again very fast. From day 12 to 15 the activity increased again, but the increase was rather modest.

The PAL activity per culture and per unit protein was on increase between days 12 and 15 i.e., the days preceding shoot differentiation.

(f) Phenolics :

Progressive changes in the phenolic content of floral bud callus during differentiation of shoots on shoot inducing medium are presented in Fig. 108 and Table 46.

The phenolic content (on culture basis) in floral bud callus increased steadily from day 0 to 9, from 0.22 mg/culture to 1.06 mg/culture. Between days 9 and 15 the phenolic content remained stable. On percentage basis, the total extractable phenolics increased from 1.87 mg% on day 0 to 3.3 mg% by day 3. Thereof till day 6 the phenolic content remained stable. Between days 6 and 15 the phenolic content was on decline, reaching the value of 2.3 mg% on day 15.

The phenolic content on percentage basis was on decline preceding shoot differentiation. Whereas on culture basis it remained more or less stable.

(g) Peroxidase Isoenzymes :

Fig. 108a illustrates progressive changes in anodic and cathodic isoperoxidases during shoot differentiation from floral bud callus.

The three initial anodic isoperoxidases A_1, A_2 and A_3 persisted right through the culture period. Anodic isoperoxidase A_4 was synthesized on day 6 and persisted till day 15. On day 12 three fast migrating anodic isoperoxidases PS_1, PS_2 and PS_3 were synthesized. These three anodic isoperoxidases were also present on day 15. The anodic isoperoxidases PS_1, PS_2 and PS_3 were also detected in the peroxidase preparation of detached regenerated shoots. Hence, the differentiation of shoots from floral bud callus was signalled prior to actual visual manifestation by above mentioned three anodic isoperoxidases.

On the cathodic scale, the initial cathodic isoperoxidases C_1 and C_2 were synthesized on all days of culture. The third initial one C_3 was synthesized only on day 3 and 6.

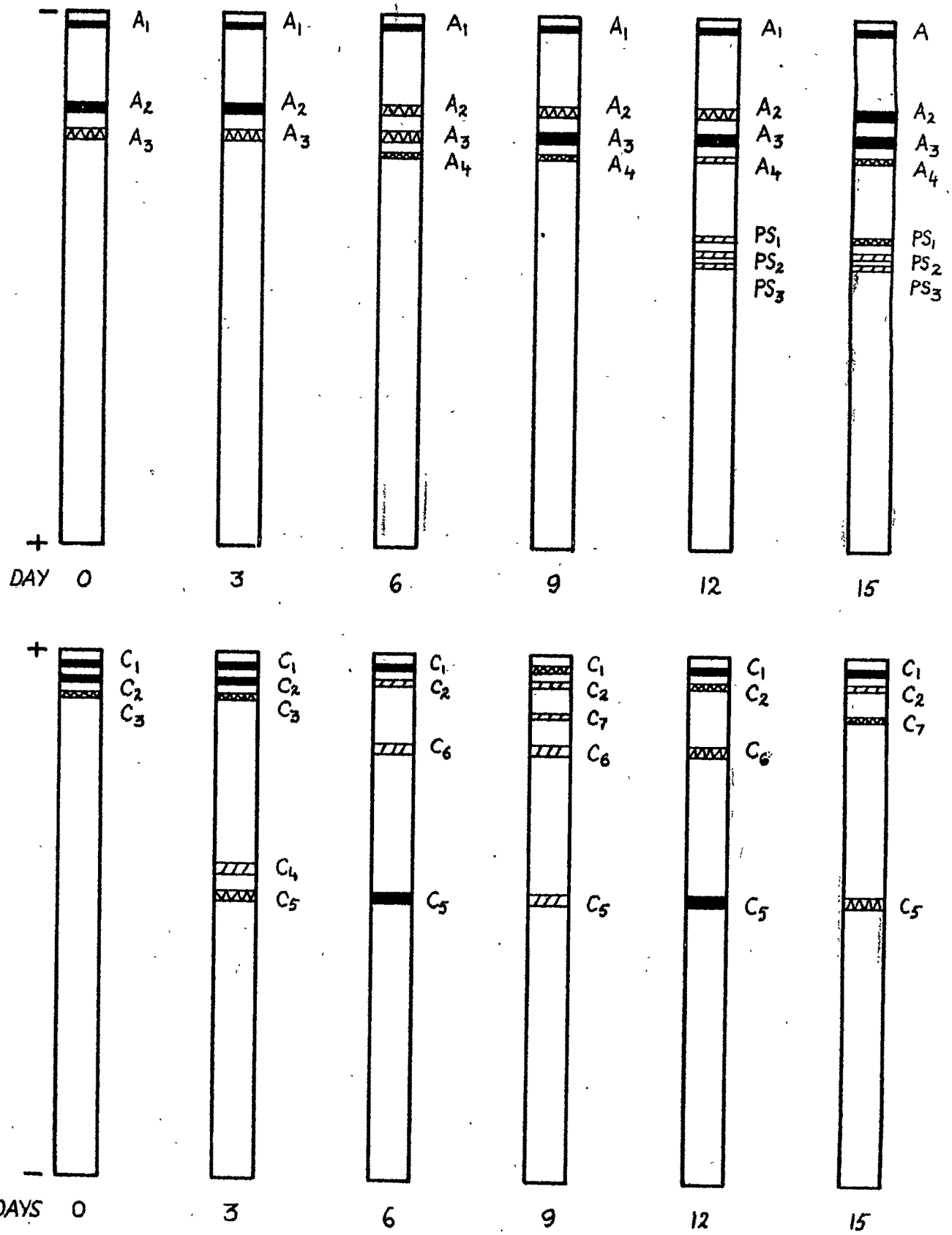


FIG. 108a.

Anodic and Cathodic Isoperoxidase Patterns.

Experimental Details as in Table 46.

For the rest of culture period, it was repressed. Two fast migrating isoperoxidases C_4 and C_5 were synthesized on day 3. Of the two C_5 persisted till day 15, whereas C_4 did not make its appearance on any other day after day 3. Two slow migrating cathodic isoperoxidases C_6 and C_7 were also synthesized. C_6 was synthesized on days 6, 9 and 12, whereas C_7 was synthesized on days 9 and 15 only.

No positive correlation could be found between the cathodic isoperoxidase banding pattern and shoot differentiation.

Expt. 35. Studies with floral bud callus cultured on shoot inducing medium supplemented with low and high levels of sucrose.

Healthy callus masses of N. tabacum weighing 300±30 mg by fresh weight were cultured on 20 ml of shoot inducing medium (MS + 0.3 mg/l IAA) in which low (1%) and high (6%) sucrose levels were added. The culture vessels were incubated at 26±2° in continuous light for a period of 15 days. Every three days 5 replicates were harvested and analysed for growth, enzymes, isoperoxidase banding patterns and phenolic content.

(a) Growth :

Growth of floral bud callus cultured on shoot inducing medium supplemented with low (1%) and high (6%) sucrose levels is illustrated in Figs. 109, 110 and Tables 47, 48.

Growth on both fresh and dry weight basis was almost linear in floral bud callus cultured on 1% sucrose containing medium. During the culture period of 15 days, fresh weight increased 3.6 fold and dry weight by 5.07 fold (Fig. 109, Table 47). On 6% sucrose medium growth of the callus on fresh weight basis was rapid between days 0 and 6. The rate of growth was rather slow thereof till day 15. Dry weight

increased rapidly between days 0-3 and days 9-12. The rate of increase in dry mass was slow between days 3-9 and 12-15. During the 15 day culture period fresh weight increased 2.35 fold and dry weight by 4.78 fold (Fig. 110, Table 48).

Compared with the standard shoot differentiating medium (MS + 0.3 mg/l IAA + 3% sucrose), both low (1%) and high (6%) sucrose levels supported better growth. On standard shoot inducing medium fresh and dry weight increases were 1.64 and 3.63 fold respectively. Furthermore, 1% sucrose in the medium supported best growth among all the three sucrose levels tested.

With the reduction of sucrose level to 1% in the standard shoot inducing medium, differentiation of shoots was completely inhibited. Use of high (6%) sucrose level in the same medium, however, brought about dramatic change in morphogenetic response. In other words, with incorporation of 6% sucrose in the medium roots were differentiated instead of shoots. These roots were differentiated any time between days 12-15, with about 25 per cent frequency.

(b) Peroxidase :

Progressive changes of peroxidase activity in floral bud callus cultured on shoot inducing medium supplemented with low (1%) and high (6%) concentrations of sucrose are illustrated in Figs. 109, 110 and Tables 47, 48.

Table : 47. Growth, Polyphenols and progressive changes in the Activity of Peroxidase, IAA Oxidase, MDH and PAL in diploid callus tissues of N. tabacum L. var. Anand-2 cultured on shoot differentiating medium supplemented with low level of sucrose.

Medium : MS + 0.3 mg/l IAA + 1% sucrose.

Incubation : 15 days at 26±2° in continuous light

Inoculum : 300±30 mg fresh tissue

Day	Fresh weight mg/cult.	Dry weight mg/cult.	PEROXIDASE		IAA OXIDASE		MDH		PAL		PHENOLICS	
			units/ cult.	units/mg protein	units/ cult.	units/mg protein	units/ cult.	units/mg protein	units/ cult.	units/mg protein	mg/ cult.	mg%
0	300 (±30)	12 (±3)	1.77	0.92	0.57	0.30	1.69	0.88	0.48	0.25	0.22	1.87
3	505.2 (±36.3)	27.2 (±1.2)	1.61	3.90	1.08	2.61	4.82	11.66	1.11	2.68	0.31	1.15
6	622.7 (±24.1)	34.2 (±2.7)	1.99	0.69	2.60	0.34	13.27	9.99	6.91	2.39	0.34	1.02
9	796.5 (±32.8)	45.1 (±3.8)	3.74	1.43	1.70	0.65	21.72	8.33	10.61	4.07	0.74	1.65
12	936.2 (±46.3)	52.8 (±3.7)	5.29	1.19	7.91	1.78	28.06	6.33	11.69	2.63	0.74	1.40
15	1078.2 (±51.9)	60.9 (±4.3)	6.79	0.85	22.45	2.82	34.40	4.33	9.57	1.20	0.53	0.87

Figures in parenthesis represent standard error.

Data represented is average of 5 replicates.

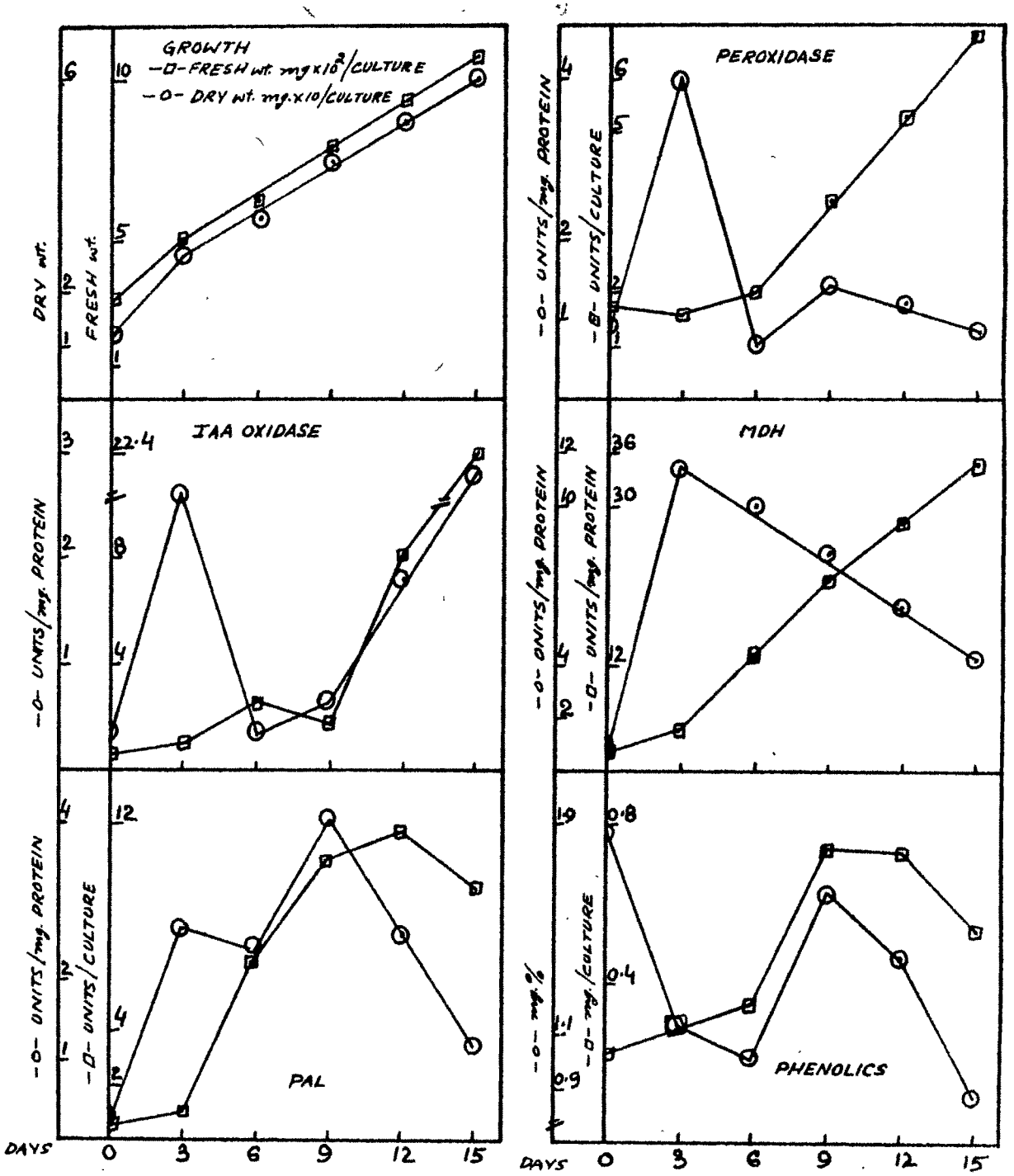


Fig. 109.

Experimental Details as in Table 47.

Table : 48. Growth, Polyphenols and progressive changes in the Activity of Peroxidase, IAA Oxidase, MDH and PAL in diploid callus tissues of N. tabacum L. var. Anand-2 cultured on shoot differentiating medium supplemented with high level of sucrose.

Medium : MS + 0.3 mg/l IAA + 6% sucrose.

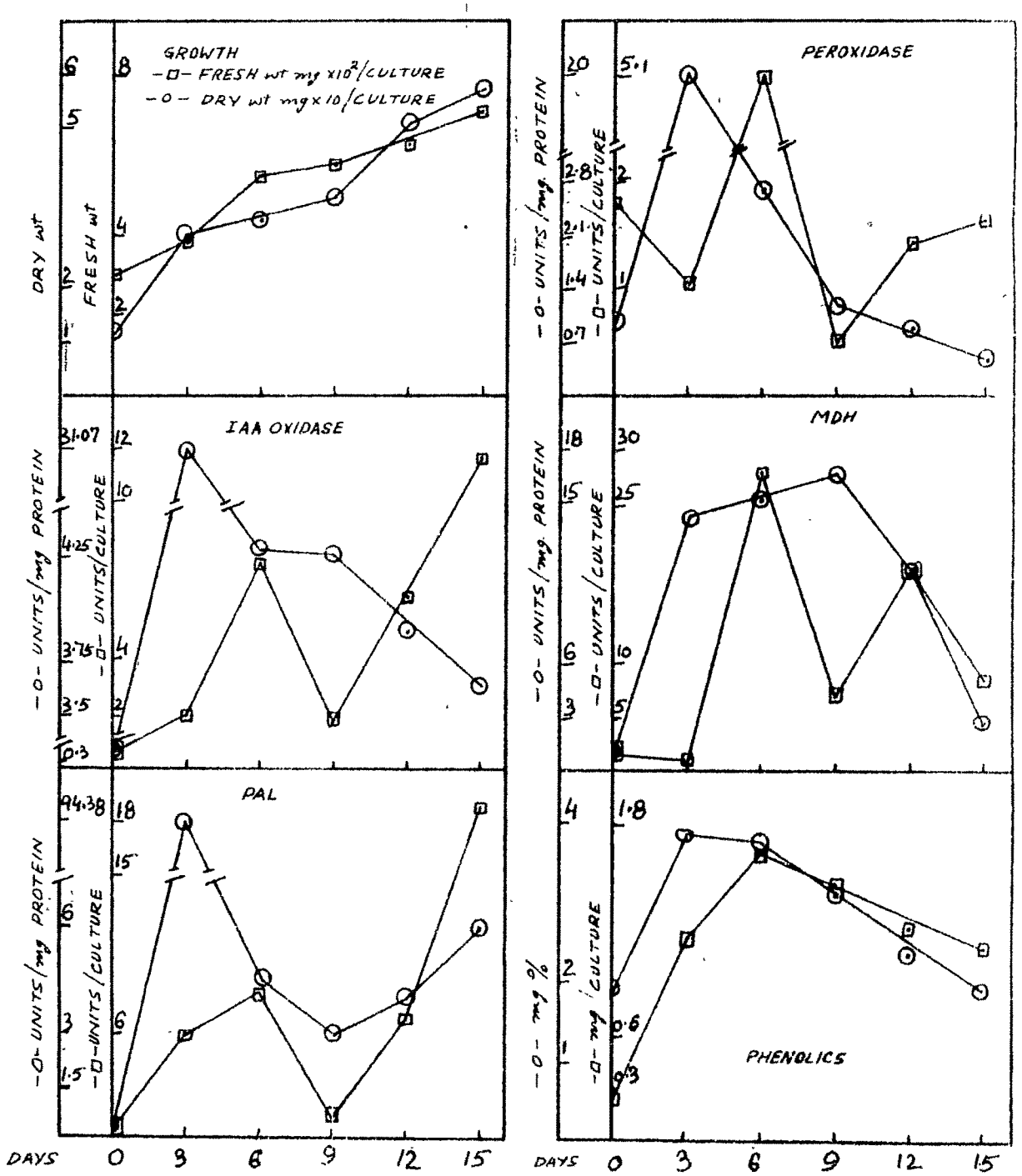
Incubation : 15 days at 26±2° in continuous light

Inoculum : 300±30 mg fresh tissue

Day	Fresh weight mg/cult.	Dry weight mg/cult.	PEROXIDASE		IAA OXIDASE		MDH		PAL		PHENOLICS	
			units/ cult.	units/mg protein	units/ cult.	units/mg protein	units/ cult.	units/mg protein	units/ cult.	units/mg protein	mg/ cult.	mg%
0	300 (±30)	12 (±3)	1.77	0.92	0.57	0.30	1.69	0.88	0.48	0.25	0.22	1.87
3	388.4 (±22.4)	30.1 (±2.3)	1.28	20.02	1.98	31.07	0.91	14.33	6.04	94.38	1.16	3.86
6	554.4 (±36.8)	43.1 (±4.1)	5.10	2.74	7.89	4.25	28.22	15.21	8.62	4.64	1.63	3.80
9	576.0 (±31.7)	46.8 (±4.8)	0.51	1.21	1.82	4.25	7.13	16.66	1.27	2.98	1.46	3.13
12	635.0 (±41.2)	51.6 (±3.9)	1.46	0.88	6.49	3.91	18.82	11.34	6.89	4.15	1.21	2.36
15	706.8 (±33.6)	57.4 (±2.9)	1.62	0.51	11.56	3.66	8.41	2.66	18.84	5.97	1.12	1.96

Figures in parenthesis represent standard error.

Data represented is average of 5 replicates.



Experimental Details as in Table 48.

FIG. 110

The total peroxidase activity of floral bud callus cultured on low (1%) sucrose containing medium declined slightly during initial three days of culture. From day 3 onwards till day 15 the enzyme activity increased linearly. Specific peroxidase activity, however, increased during the initial three days of culture. The peak activity was attained on day 3. Between days 3-6 the activity declined sharply. However, from day 6 to 9, a modest increase in the activity ensued, only to decay further thereof till day 15 (Fig. 109, Table 47).

Total peroxidase activity of floral bud callus cultured on high (6%) sucrose containing medium declined appreciably between days 0 and 3. The activity, however, increased by day 6 to its first peak value. By day 9 peroxidase activity declined again to touch the lowest value during the entire culture period. From day 9 till day 15 the activity was on increase again and reached its second peak value on day 15. Specific peroxidase activity increased between day 0 and 3 to reach peak value. Thereof it declined till day 15 (Fig. 110, Table 48).

In contrast with increasing peroxidase activity per unit protein during last phase of culture (day 12-15) of floral bud callus on standard shoot inducing medium (whereon shoots were differentiated on day 15), the peroxidase activity

per unit protein was on decline during the same phase of culture (day 12-15), when low (1%) or high (6%) sucrose level was used.

(c) IAA Oxidase :

Progressive changes in the activity of IAA Oxidase in floral bud callus cultured on shoot inducing medium supplemented with low (1%) and high (6%) sucrose levels, are presented in Figs. 109, 110 and Tables 47, 48.

Total IAA Oxidase activity in the callus cultured on 1% sucrose containing medium increased during the first six days of culture. Between days 6 and 9 the enzyme activity declined, but increased again from day 9 till day 15. Specific IAA Oxidase activity exhibited double-peak developmental pattern. The activity increased sharply between days 0 and 3 reaching its first peak value. Rapid decay in activity ensued between days 3 and 6. However, it again started to increase thereon and continued to do so till day 15, when second peak value was reached (Fig. 109, Table 47).

As in the previous case, total IAA Oxidase activity of floral bud callus cultured on 6% sucrose medium increased between days 0 and 6, reaching its first peak value on day 6. On that particular day the activity was about three times .

higher than in callus tissues cultured on 1% sucrose medium. The enzyme activity declined between days 6 and 9, but climbed up again from day 9 onwards to reach peak activity on day 15. Specific IAA Oxidase activity reached its peak value on day 3 subsequent to rapid increase in activity. The activity on that particular day was about 12 times more than the peak value on the same day in callus tissues cultured on 1% sucrose medium. The specific IAA Oxidase activity decayed rapidly between days 3 and 6; remained stable between days 6 and 9, and from thereon continued to decline linearly till day 15 (Fig. 110, Table 48).

The total IAA Oxidase activity per unit protein was on increase in callus tissues cultured on 1% sucrose medium between days 9 and 15. On 6% sucrose medium during the corresponding period the activity was on decline. On standard shoot inducing medium (MS + 0.3 mg/l IAA) which contained 3% sucrose the activity decayed between day 9 and 15. Though IAA Oxidase activity per unit protein was on decline during the final phase of culture (day 9-15) in floral bud callus cultured on standard shoot inducing medium as well as on 6% sucrose containing medium, the decline in activity was more rapid on standard shoot forming medium (from 4.3 units/mg protein to 2.4 units/mg protein) than on 6% sucrose containing medium (from 4.25 units/mg protein to 3.63 units/mg protein).

On the former medium shoots were differentiated on day 15 and on the latter root differentiation occurred between days 12-15. No organogenesis occurred on 1% sucrose medium.

(d) MDH :

Progressive changes of MDH activity in floral bud callus cultured on shoot inducing medium (MS + 0.3 mg/l IAA) supplemented with 1% and 6% sucrose, are illustrated in Figs. 109, 110 and Tables 47, 48.

In callus cultured on 1% sucrose containing medium, total MDH activity increased from day 0 till day 15. The rate of increase of activity was rather slow during the initial three days of culture, but thereof it increased linearly and rapidly till day 15. Specific MDH activity on the same medium increased sharply between days 0 and 3, reaching its peak value. Between days 3 and 15 the activity was continuously on decline along a linear slope (Fig. 109, Table 47).

Total MDH activity in floral bud callus cultured on 6% sucrose medium decayed marginally between days 0 and 3. It, however, increased over 30 fold between days 3 and 6 to attain its peak value on day 6. The activity declined again between days 6 and 9. Thereof it increased to reach the second peak value on day 12. By day 15 the enzyme activity had decayed again. Specific MDH activity increased sharply between days

O and 3. The activity continued to increase between days 3 and 9, but the rate of increase was very slow. The enzyme activity decayed very rapidly between days 9 and 15 (Fig.110, Table 48).

On the control medium (shoot inducing MS medium + 0.3 mg/l IAA + 3% sucrose) the MDH activity per unit protein was on linear increase between days 9-15 and shoots were differentiated on day 15. On incorporation of low (1%) and high (6%) sucrose into the same medium the MDH activity per unit protein during the same period (day 9-15) was on decline. The decay of activity was much rapid on 6% sucrose medium (16.7 units/mg protein to 2.7 units/mg protein) that on 1% sucrose medium (8.2 units/mg protein to 4.2 units/mg protein). Roots were differentiated on 6% sucrose medium, whereas, organogenesis was completely inhibited with the addition of 1% sucrose.

(e) PAL :

Progressive changes of PAL activity in floral bud callus cultured on shoot inducing medium containing 1% and 6% sucrose are illustrated in Figs. 109, 110 and Tables 47, 48.

PAL activity in callus on 1% sucrose medium increased slowly between days O and 3. It was followed by sharp increase between days 3 and 12. The peak value was reached on

day 12, from thereon till day 15 the enzyme activity declined. Specific PAL activity in floral bud callus on the same medium increased rather rapidly till day 3. By day 6 the enzyme activity had declined a little, but increased again thereof to attain peak value on day 9. Between days 9 and 15 the activity declined linearly and sharply (Fig. 109, Table 47).

On 6% sucrose containing medium PAL activity increased between days 0 and 6, reaching first peak value. Between days 6 and 9 the enzyme activity declined, whereof it increased again till day 15 reaching the second peak value during the culture period. Specific PAL activity on the same medium increased sharply - about 380 fold - during the first three days of culture. The activity, however, declined very rapidly between days 3 and 9. Between days 9 and 15 it was again on increase (Fig. 110, Table 48).

On the standard shoot inducing medium PAL activity per unit protein exhibited a very modest increase in activity between days 12 and 15 i.e., the days immediately preceding shoot differentiation. During the corresponding period PAL activity on 1% sucrose medium was rapidly declining; whereas on 6% sucrose medium it was on increase. Though PAL activity per unit protein increased on both, standard shoot inducing medium and the same medium containing 6% sucrose, the activity on the former medium was 25 to 30 times less than on the

latter medium whereon roots were differentiated.

(f) Phenolics :

Changes in phenolic content of floral bud callus during its growth on shoot inducing medium supplemented with low (1%) and high (6%) sucrose, are presented in Figs. 109, 110 and Tables 47, 48.

Phenolic content in callus tissues cultured on 1% sucrose medium increased modestly from 0.22 mg/culture on day 0 to 0.34 mg/culture on day 6. By day 9 phenolic accumulation had increased to 0.74 mg/culture and remained stable till day 12. By day 15 the phenolic content had declined to 0.53 mg/culture. On percentage basis phenolic content declined from 1.87 mg% on day 0 to 1.02 mg% on day 6. By day 9 it, however, increased to 1.65 mg%, but declined again thereof till day 15 to the value of 0.87 mg% (Fig. 109, Table 47).

The phenolic content in callus cultured on shoot inducing medium containing 6% sucrose, increased from 0.22 mg/culture on day 0 to 1.63 mg/culture by day 6. The phenolic content, however, declined linearly between days 6 and 15 to 1.12 mg/culture. On percentage basis the phenolic content increased from 1.87 mg% on day 0 to 3.86 mg% on day 3. By day 6 the phenolic content decreased a little to 3.80 mg%, but thereof decline was much more pronounced and almost linear till day 15. On day 15 phenolic content had dropped

to 1.96 mg% (Fig. 110, Table 48).

Phenolic content on both 1% and 6% sucrose media was on decline during the last phase of culture (days 12 to 15). On 1% sucrose medium whereon organogenesis was completely inhibited the phenolic content declined from 1.4 mg% to 0.87 mg% between days 12 and 15. On 6% sucrose medium whereon root differentiation occurred (between days 12-15) the phenolics during the same period (days 12-15) declined from 2.4 mg% to 1.96 mg%. On the other hand, on the standard shoot differentiating medium which was supplemented with 3% sucrose, phenolic content declined from 2.8 mg% to 2.3 mg% during the corresponding period.

(g) Peroxidase Isoenzymes :

Progressive changes of peroxidase isoenzyme banding pattern in floral bud callus cultured on shoot inducing medium supplemented with low (1%) and high (6%) sucrose levels is illustrated in Fig. 109a and 110a.

In callus tissues cultured on 1% sucrose containing medium all three initial anodic isoperoxidases A_1, A_2 and A_3 remained repressed on days 3 and 6. A_1, however, reappeared between days 9-15, and A_2 was synthesized on day 12. A_3 remained suppressed for the entire culture period. Five slow migrating isoperoxidases A_4-A_8 were synthesized during the

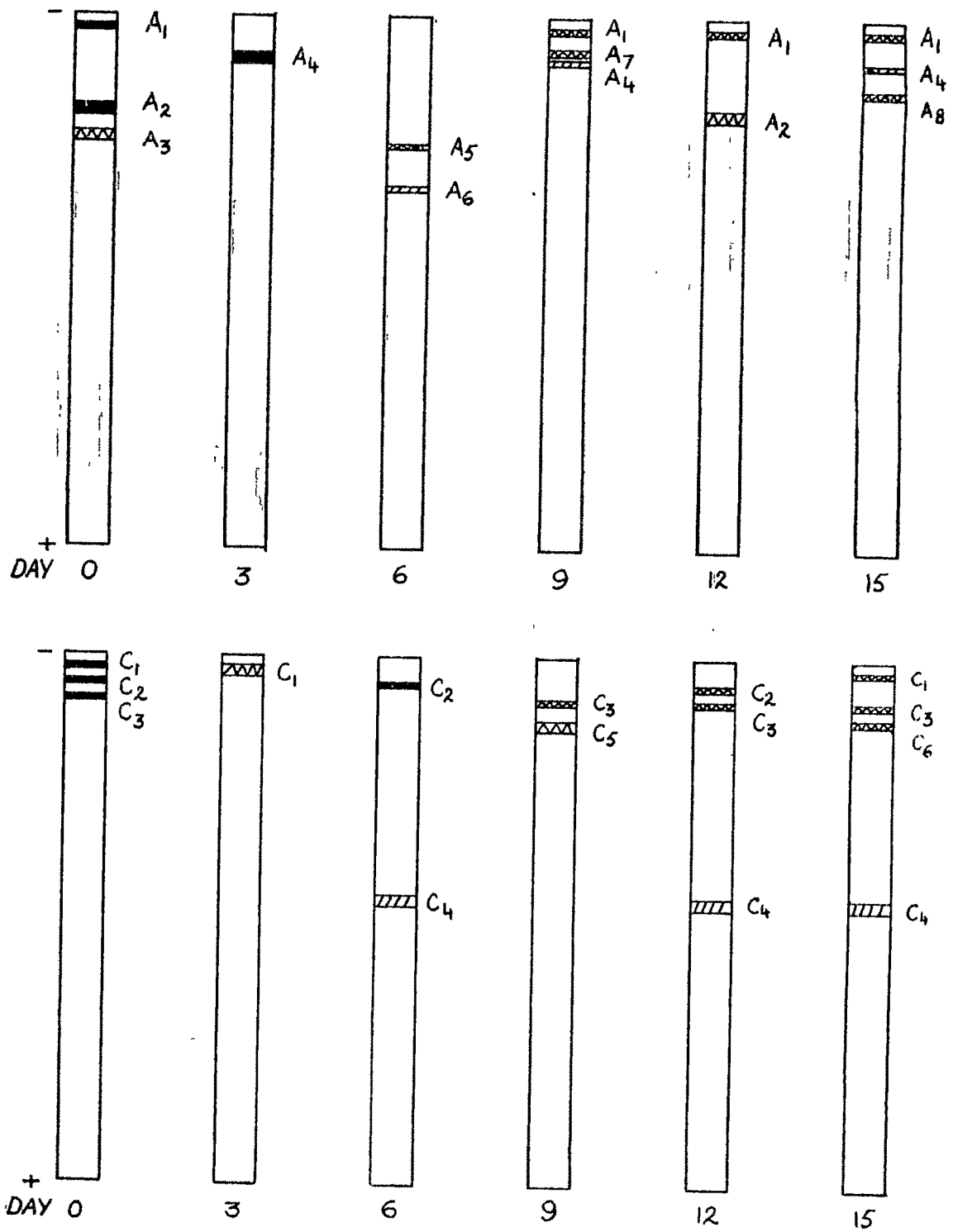
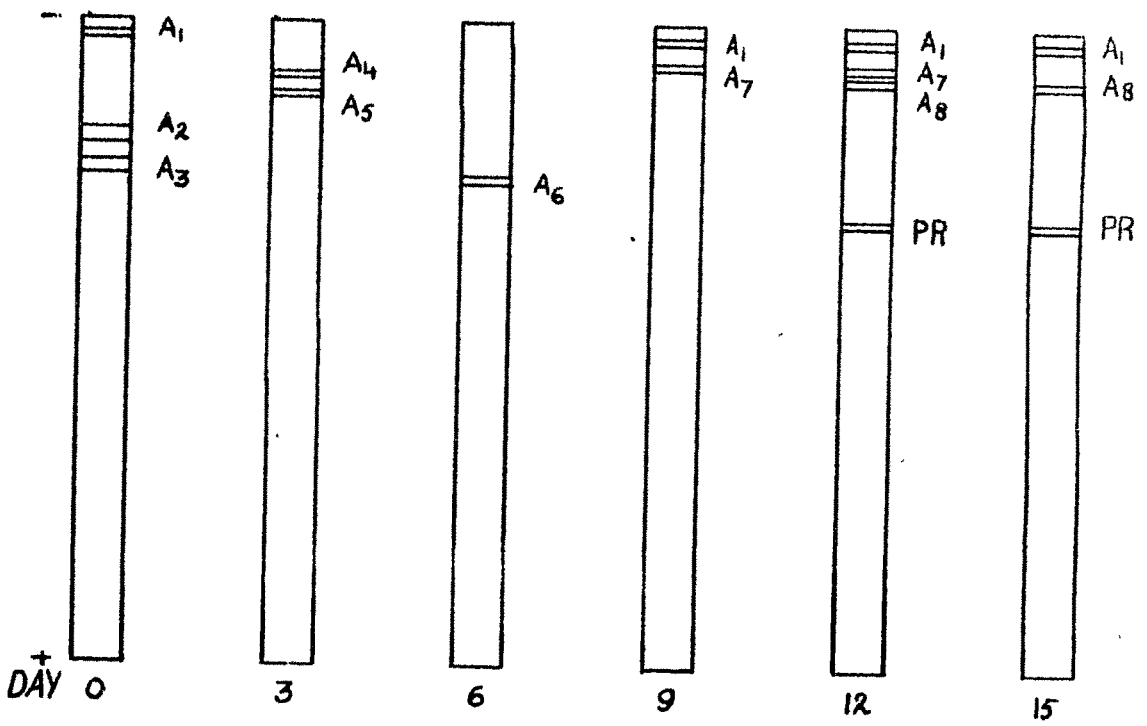
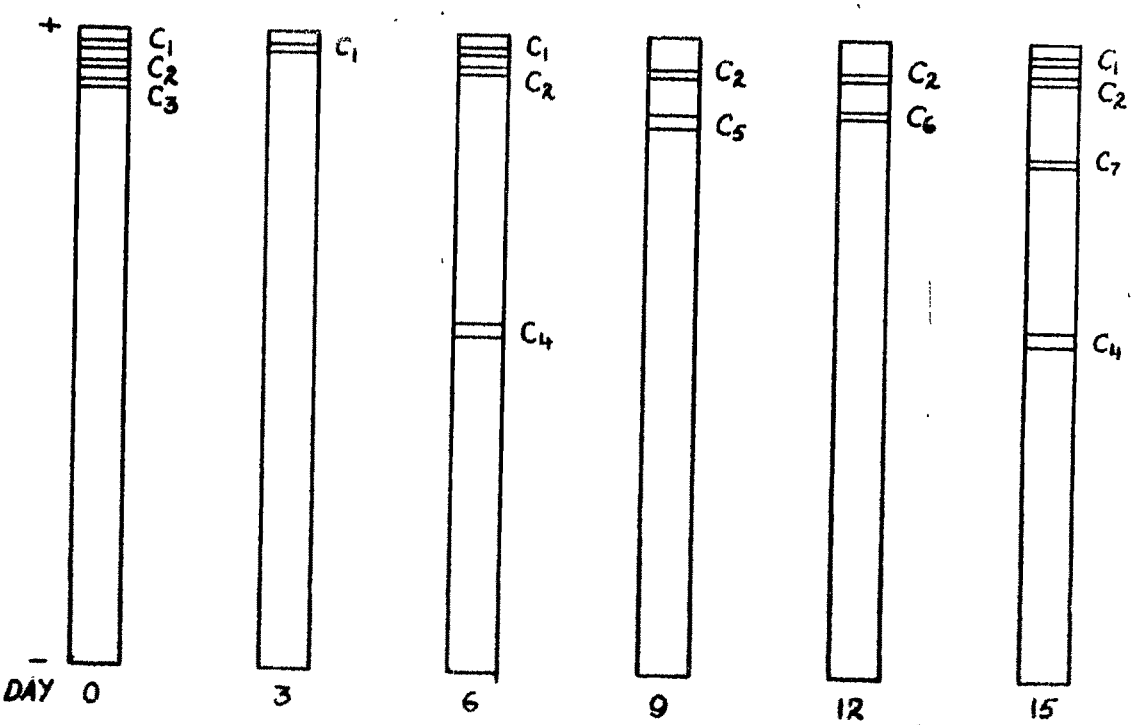


Fig. 109a

Anodic and Cathodic Isoperoxidase Patterns.

Experimental Details as in Table 47.





Anodic and Cathodic Isoperoxidase Patterns.
Experimental Details as in Table 48

Fig. 110 a.

culture period. A_4 was synthesized on days 3, 9 and 15, A_5 and A_6 on day 6, A_7 on day 12 and A_8 on day 15. The shoot isoperoxidases PS_1, PS_2 and PS_3 were not synthesized on this 1% sucrose containing shoot inducing medium. On the cathodic scale, of the three initial cathodic isoperoxidases C_1 appeared on days 3 and 15, C_2 on days 6 and 9, and C_3 on days 9, 12 and 15. Slow migrating cathodic isoperoxidase C_5 on day 9 and C_6 on day 12 were also synthesized. A fast migrating cathodic isoperoxidase C_4 appeared on days 6, 12 and 15 (Fig. 109a).

On 6% sucrose containing shoot inducing medium the anodic isoperoxidases A_1, A_2 and A_3 were not synthesized on days 3 and 6. Isoperoxidases A_2 and A_3 were not synthesized even on the subsequent days of culture. A_1, however, appeared between days 9-15. During the culture period five slow migrating anodic isoperoxidases A_4-A_5 were synthesized. A_4 and A_5 were synthesized on day 3, A_6 on day 6, A_7 on days 9 and 12, and A_8 on days 12 and 15. On day 12 preceding root differentiation, the root isoperoxidase "PR" was synthesized and it persisted on day 15. Of significance was the absence of shoot isoperoxidases PS_1, PS_2 and PS_3. These three isoperoxidases, as was demonstrated in earlier experiment, precede shoot differentiation from floral bud callus. On the cathodic scale the initial cathodic isoperoxidase C_3

remained suppressed for the entire length of culture period. Other initial isoperoxidases C_1 and C_2 were synthesized on days 3, 6 and 15, and days 6-15 respectively. Three slow migrating isoperoxidases C_5 on day 9, C_6 on day 12 and C_7 on day 15 were also synthesized. A fast migrating cathodic isoperoxidase C_4 was synthesized on days 6 and 9 (Fig. 110a).

Expt. 36. Studies with floral bud callus cultured on shoot inducing medium supplemented with various phenolic acids.

Healthy callus masses weighing 300±30 mg by fresh weight were cultured on 20 ml of shoot inducing medium (MS basal + 0.3 mg/l IAA + 3% sucrose) into which various phenolic acids were supplemented individually in the concentrations enlisted below :

(a) t-cinnamic acid (100 μM)
(b) t-cinnamic acid (500 μM)
(c) caffeic acid (100 μM)
(d) caffeic acid (500 μM)
(e) ferulic acid (100 μM)
(f) ferulic acid (500 μM)
(g) p-hydroxybenzoic acid (1.0 μM)
(h) p-hydroxybenzoic acid (10.0 μM)

The culture vessels were incubated at 26±2° in continuous light for a period of 15 days. Every three days a fixed number of 5 replicates was harvested and analysed. for growth, enzymes, isoperoxidase banding pattern and phenolic accumulation.

(a) Growth :

Growth, expressed as increase in fresh and dry weights

of floral bud callus on the shoot forming medium (MS basal + 0.3 mg/l IAA + 3% sucrose) into which the phenolic acids were added are illustrated in Figs. 111-118 and Tables 49-56.

Growth on both fresh and dry weight basis was stimulated with the addition of phenolic acids into the medium. Maximum fresh weight increament of 5.33 fold occurred on medium supplemented with 500 μM caffeic acid. Maximum dry weight increase of 13.28 fold was recorded on 100 μM ferulic acid medium.

With the incorporation of 100 μM t-cinnamic acid (a) into the medium, the fresh weight increased 4.78 folds and the dry weight by 9.46 folds (Fig. 111, Table 49). With the increase of t-cinnamic acid concentration to 500 μM (b), the increases in the fresh and dry weights were somewhat lesser than in the previous instance. The fresh weight increased 4.46 fold and the dry weight 7.95 fold (Fig. 112, Table 50).

Addition of 500 μM caffeic acid into the medium proved to be better growth promoter than 100 μM caffeic acid. The increase in fresh weight on 100 (c) and 500 μM (d) caffeic acid containing media were, 4.67 and 5.33 fold respectively. Similarly the dry weight on the two media increased 10.14 and 11.76 fold respectively (Figs. 113, 114, Tables 51, 52).

Ferulic acid at 100 μM (e) promoted better growth

than at 500 μM (f) concentration. The fresh weights on the two media increased 5.24 and 4.36 fold respectively. Corresponding dry weight increases were 13.28 and 10.5 fold (Figs. 115, 116; Tables 53, 54).

Though the incorporation of 1.0 μM p-hydroxybenzoic acid (g) into the medium supported better growth than 10.0 μM p-hydroxybenzoic acid (h), the differences in growth values were not significant. The respective fresh weight increases on the two media were 4.36 and 4.33 fold. The corresponding dry weight increases were 9.3 and 9.15 folds (Figs. 117, 118; Tables 55, 56).

Addition of all the above mentioned phenolic acids into the MS medium containing 0.3 mg/l IAA and 3% sucrose completely suppressed differentiation of shoots.

(b) Peroxidase

Progressive changes of peroxidase activity in floral bud callus cultured on MS basal medium + IAA (0.3 mg/l) + sucrose (3%) and supplemented with two levels each of the above mentioned four phenolic acids, are illustrated in Figs. 111-118 and Tables 49-56.

The peroxidase activity in the callus cultured on 100 μM t-cinnamic acid (a) containing medium declined

slightly by day 3. Thereon till day 15 it increased steadily to reach its peak value. The specific activity of peroxidase on the same medium declined by day 3 but increased again to attain two peaks, one each on days 6 and 15 (Fig. 111, Table 49). On 500 μM t-cinnamic acid (b) medium the peroxidase activity as in the previous instance declined till day 3. Thereon it increased to its peak value on day 9. Between days 9 and 15 the enzyme activity declined slowly. Between days 0 and 3 the specific activity of peroxidase decayed. It, however, increased between days 3 and 9, before registering sharp decline in the activity between days 9 and 15 (Fig. 112, Table 50).

With the incorporation of 100 μM t-cinnamic acid into the shoot inducing medium the peroxidase activity per unit protein was 12 to 15 per cent higher between days 12 and 15 as compared with peroxidase activity in floral bud callus during the same period, when shoots were differentiated on standard shoot inducing medium. With the increase of t-cinnamic acid level to 500 μM in the same medium, during the corresponding period (i.e. days 12 and 15) the activity of peroxidase was declining and was 2 to 4 fold less as compared with the activity on the standard shoot inducing medium. Incorporation of t-cinnamic acid (100, 500 μM) into the standard shoot inducing medium completely suppressed caulogenic response.

The addition of 100 μM caffeic acid (c) induced a double peaked pattern in peroxidase activity. Prior to attaining peak values, one each on days 6 and 15, the enzyme activity declined between days 0 and 3. The specific activity of peroxidase on the same medium before attaining peak activity on day 6 decayed between days 0 and 3. Between days 6 and 15 the enzyme activity declined sharply (Fig. 113, Table 51). On 500 μM caffeic acid (d) medium the peroxidase activity after declining between days 0 and 3, increased steadily to its peak value on day 15. The specific activity of peroxidase too, on this medium declined slightly by day 3. The peak value attained on day 9 was followed by a sharp decline in the specific activity of peroxidase till day 15 (Fig. 114, Table 52).

Contrary to the peroxidase activity per unit protein in floral bud callus cultured on standard shoot inducing medium, the peroxidase activity was on decline between days 12 and 15, with the incorporation of caffeic acid (100, 500 μM) into the medium. At both the levels of caffeic acid the peroxidase activity on day 12 was higher than the control, but on day 15 it was about 50 per cent of the control. Caffeic acid also inhibited shoot differentiation.

On 100 μM ferulic acid (e) medium the peroxidase enzyme activity decreased between days 0 and 3. Thereafter

the enzyme activity increased to attain two peaks, one each on days 6 and 15. The specific activity of peroxidase on this medium decreased between days 0 and 3. Thenof it increased to its peak value on day 6, followed by sharp decline in the activity till day 9. Between days 9 and 15 though the specific activity of peroxidase continued to decline, however, the decay was rather slow (Fig. 115, Table 53). The peroxidase activity in floral bud callus on 500 μM ferulic acid (f) increased steadily between days 0 and 12 to reach its peak value. The activity, however, declined sharply between days 12 and 15. The specific activity of peroxidase on the same medium increased slowly and linearly till day 6. Between days 6 and 9 the activity increased sharply to reach its peak value. Between days 9 and 15 the specific peroxidase activity decayed rapidly (Fig. 116, Table 54).

As was the case with the incorporation of caffeic acid into the shoot inducing medium, here too, with the use of ferulic acid (100, 500 μM) into the shoot inducing medium, the peroxidase activity per unit protein was on decline between days 12 and 15 in floral bud callus. During the same period (i.e. days 12 to 15) the enzyme activity on standard shoot inducing medium was higher and increasing. Incorporation of ferulic acid too, at both the levels, suppressed shoot differentiation.

The peroxidase activity in callus cultured on shoot inducing medium supplemented with 1.0 μM p-hydroxybenzoic acid (g) declined till day 3. Thereafter the activity increased to reach its peak value on day 9. The specific peroxidase activity also dropped a little by day 3, but thereof it climbed to its peak value on day 15 (Fig. 117, Table 55). The peroxidase activity declined till day 3 in callus cultured on 10.0 μM p-hydroxybenzoic acid medium (h). From thereon it increased attaining two peak values, one each on days 9 and 15. The specific peroxidase activity on this medium did not decline between days 0 and 3, but from day 0 till day 9 it increased to its peak value. The activity declined sharply between days 9 and 12. It, nevertheless, increased again by day 15 (Fig. 118, Table 56).

With the incorporation of p-hydroxybenzoic acid (1.0, 10.0 μM) into the shoot inducing medium, the peroxidase activity per unit protein increased between days 12 and 15. Similarly, the enzyme activity during the corresponding period increased in floral bud callus cultured on standard shoot inducing medium. However, on both the days i.e. days 12 and 15, the enzyme activity on p-hydroxybenzoic acid containing media was two to two and a half times more than the control. As p-hydroxybenzoic acid too inhibited shoot differentiation.

Table : 49. Growth, Polyphenols and progressive changes in the Activity of Peroxidase, IAA Oxidase, MDH and PAL in diploid callus tissues of N. tabacum L. var. Anand-2 cultured on shoot differentiating medium supplemented with 100uM t-cinnamic acid.

Medium : MS + 0.3 mg/l IAA + 100 uM t-cinnamic acid + 3% sucrose.

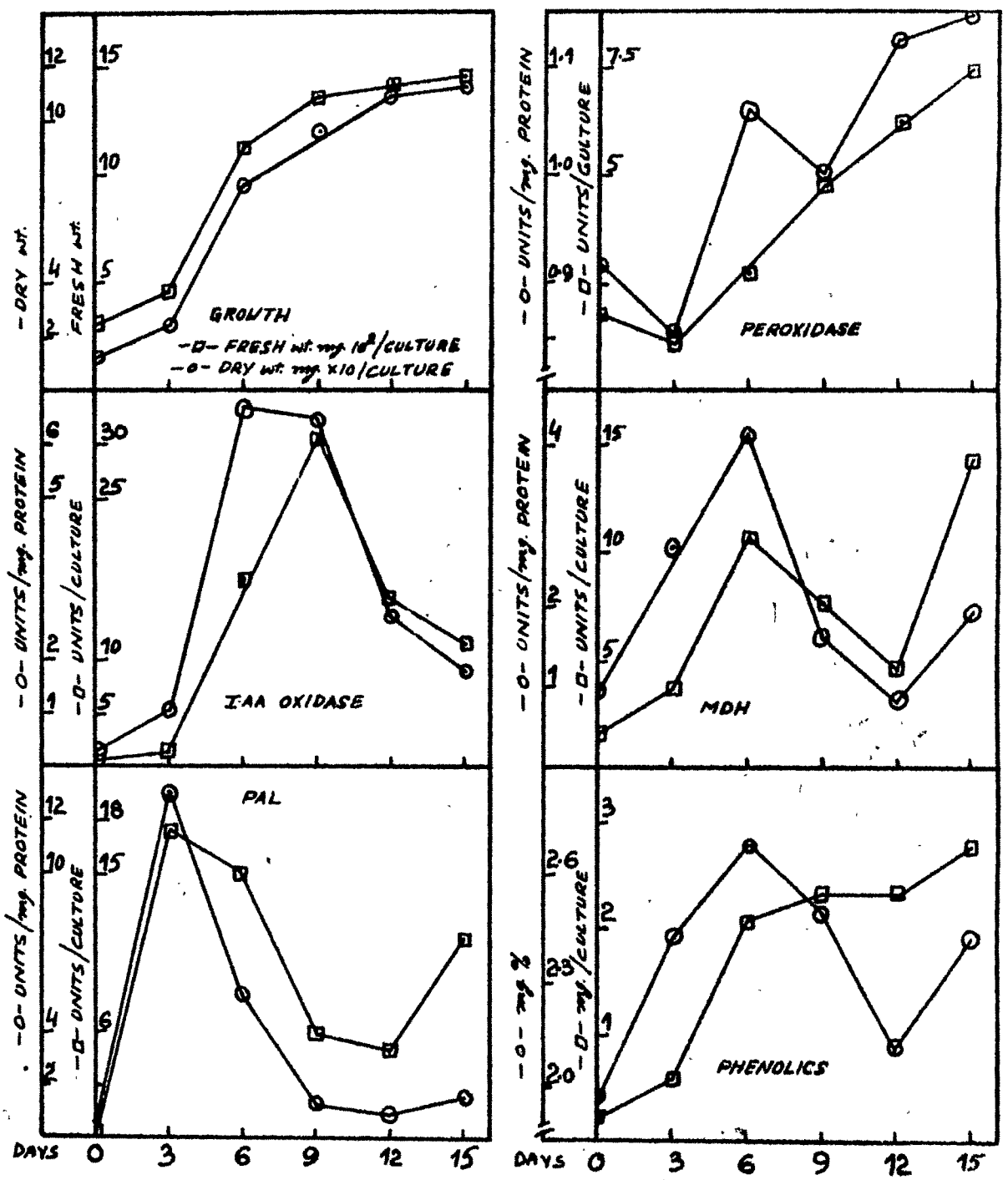
Incubation : 15 days at 26±2° in continuous light

Inoculum : 300±30 mg fresh tissue

Day	Fresh weight mg/cult.	Dry weight mg/cult.	PEROXIDASE		IAA OXIDASE		MDH		PAL		PHENOLICS	
			units/ cult.	units/mg protein	units/ cult.	units/mg protein	units/ cult.	units/mg protein	units/ cult.	units/mg protein	mg/ cult.	mg%
0	300 (±30)	12 (±3)	1.77	0.92	0.57	0.30	1.69	0.88	0.48	0.25	0.22	1.87
3	463.7 (±28.6)	24.6 (±2.2)	1.14	0.85	1.40	1.04	3.70	2.75	17.51	12.98	0.59	2.43
6	1126.6 (±80.1)	77.5 (±3.8)	2.79	1.06	17.62	6.74	10.81	4.14	15.01	5.75	2.07	2.68
9	1363.5 (±76.3)	93.8 (±6.5)	4.71	1.00	30.75	6.52	7.63	1.62	6.05	1.28	2.34	2.50
12	1419.6 (±74.9)	110.3 (±11.2)	6.24	1.12	15.83	2.85	4.55	0.82	5.11	0.92	2.33	2.12
15	1462.1 (±82.7)	113.6 (±9.8)	7.39	1.15	11.63	1.81	14.19	1.91	11.33	1.53	2.76	2.43

Figures in parenthesis represent standard error.

Data represented is average of 5 replicates.



Experimental Details as in Table. 49.

FIG. 111.

Table :50. Growth, Polyphenols and progressive changes in the Activity of Peroxidase, IAA Oxidase, MDH and PAL in diploid callus tissues of N. tabacum L. var. Anand-2 cultured on shoot differentiating medium supplemented with 500 uM t-cinnamic acid.

Medium : MS + 0.3 mg/l IAA + 500 uM t-cinnamic acid + 3% sucrose.

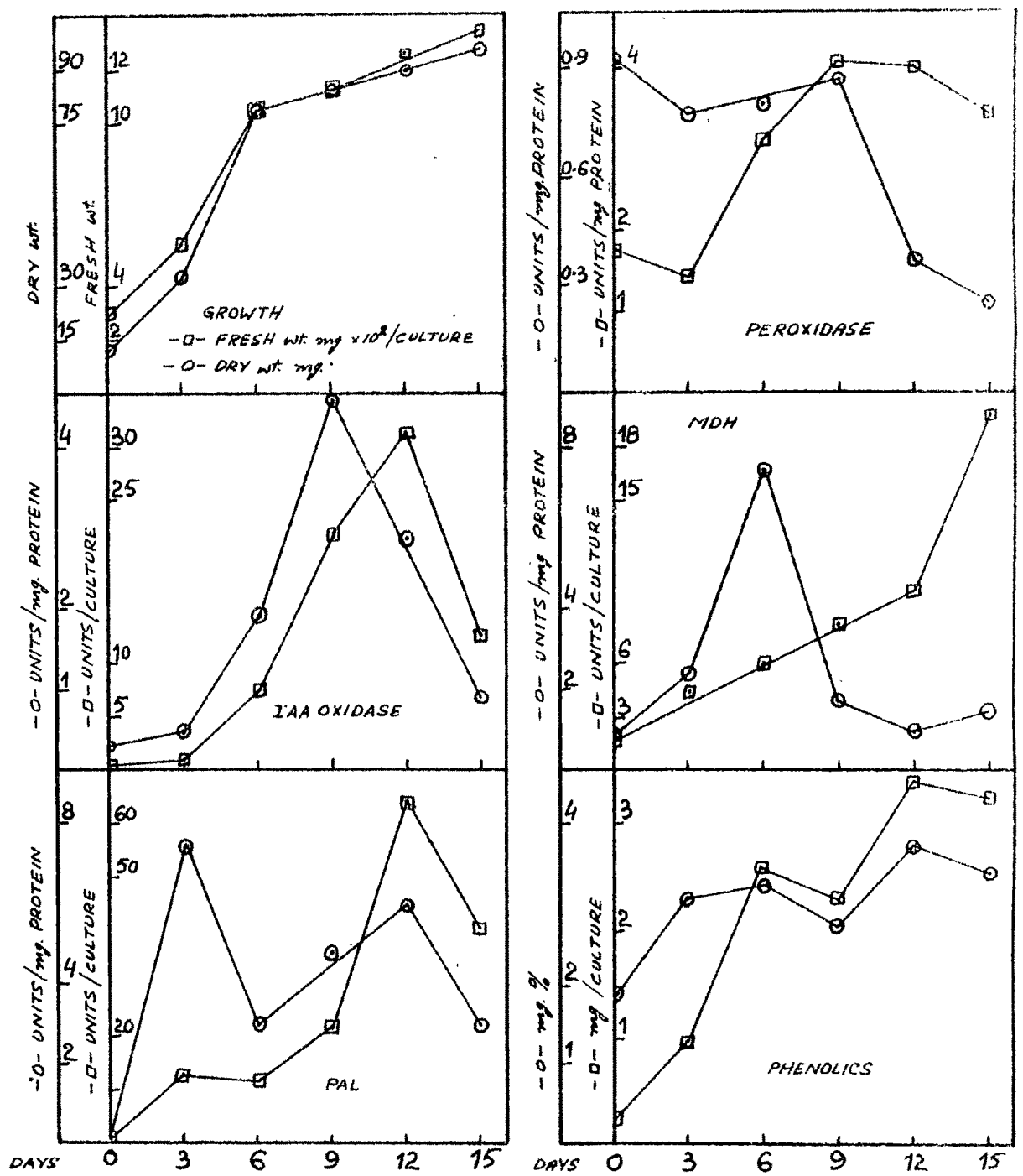
Incubation : 15 days at 26±2° in continuous light

Inoculum : 300±30 mg fresh tissue

Day	Fresh weight mg/cult.	Dry weight mg/cult.	PEROXIDASE		IAA OXIDASE		MDH		PAL		PHENOLICS	
			units/ cult.	units/mg protein	units/ cult.	units/mg protein	units/ cult.	units/mg protein	units/ cult.	units/mg protein	mg/ cult.	mg%
0	300 (±30)	12 (±3)	1.77	0.92	0.57	0.30	1.69	0.88	0.48	0.25	0.22	1.87
3	557.6 (±25.4)	32.3 (±2.7)	1.41	0.77	0.87	0.48	4.46	2.44	13.62	7.46	0.98	3.06
6	1073.3 (±50.1)	79.8 (±4.8)	3.15	0.79	7.50	1.90	6.01	7.52	11.94	3.01	2.61	3.28
9	1119.2 (±87.7)	83.3 (±4.3)	4.05	0.86	21.89	4.67	8.05	1.72	22.38	4.78	2.28	2.74
12	1265.6 (±66.2)	90.3 (±6.1)	3.99	0.37	31.39	2.91	9.97	0.92	64.79	6.02	3.37	3.74
15	1338.2 (±82.5)	95.5 (±3.7)	3.43	0.24	12.75	0.91	19.88	1.43	41.61	2.99	3.23	3.39

Figures in parenthesis represent standard error.

Data represented is average of 5 replicates.



Experimental Details as in Table 50.

FIG. 112

Table :51. Growth, Polyphenols and progressive changes in the Activity of Peroxidase, IAA Oxidase, MDH and PAL in diploid callus tissues of N. tabacum L. var. Anand-2 cultured on shoot differentiating medium supplemented with 100 uM caffeic acid.

Medium : MS + 0.3 mg/l IAA + 100 uM caffeic acid + 3% sucrose

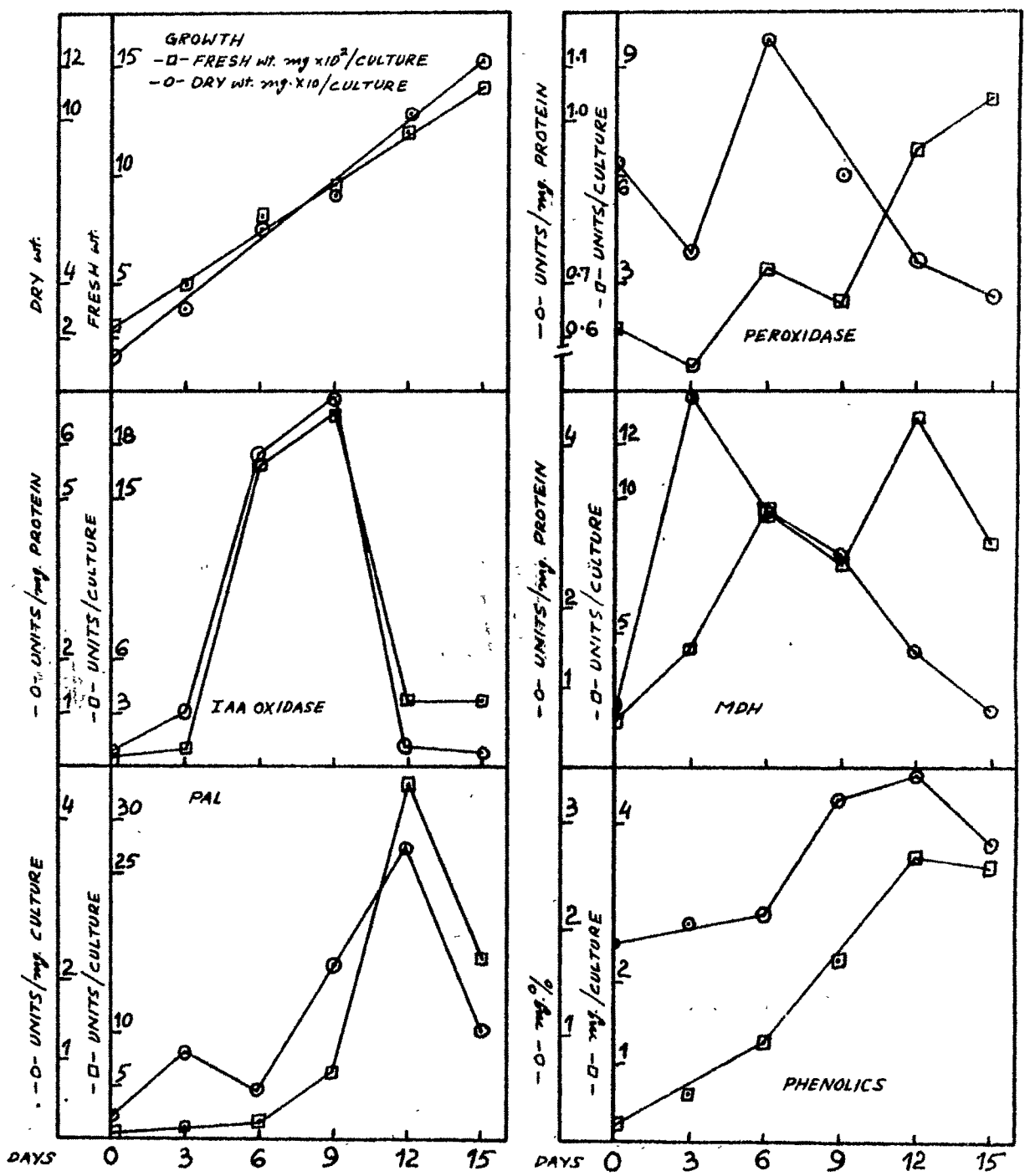
Incubation : 15 days at 26±2° in continuous light

Inoculum : 300±30 mg fresh tissue

Day	Fresh weight mg/cult.	Dry weight mg/cult.	PEROXIDASE		IAA OXIDASE		MDH		PAL		PHENOLICS	
			units/ cult.	units/mg protein	units/ cult.	units/mg protein	units/ cult.	units/mg protein	units/ cult.	units/mg protein	mg/ cult.	mg%
0	300 (±30)	12 (±3)	1.77	0.92	0.57	0.30	1.69	0.88	0.48	0.25	0.22	1.87
3	496.3 (±24.9)	30.3 (±2.7)	0.75	0.76	1.03	1.04	4.56	4.60	1.10	1.11	2.62	2.05
6	802.9 (±60.3)	60.2 (±4.0)	3.40	1.15	17.16	5.80	9.63	3.25	1.78	0.60	1.27	2.12
9	958.2 (±55.9)	71.9 (±3.9)	2.60	0.90	19.95	6.94	7.66	2.66	6.38	2.22	2.31	3.22
12	1191.9 (±81.6)	103.4 (±4.8)	6.81	0.74	3.74	0.41	13.05	1.43	33.59	3.69	3.57	3.46
15	1403.6 (±87.4)	121.7 (±5.1)	8.13	0.68	3.72	0.31	8.49	0.71	16.93	1.42	3.43	2.82

Figures in parenthesis represent standard error.

Data represented is average of 5 replicates.



Experimental Details as in Table 51.

FIG. 119.

Table: 52. Growth, Polyphenols and progressive changes in the Activity of Peroxidase, IAA Oxidase, MDH and PAL in diploid callus tissues of N. tabacum L. var.Anand-2 cultured on shoot differentiating medium supplemented with 500 uM caffeic acid.

Medium : MS + 0.3 mg/l IAA + 500 uM caffeic acid + 3% sucrose

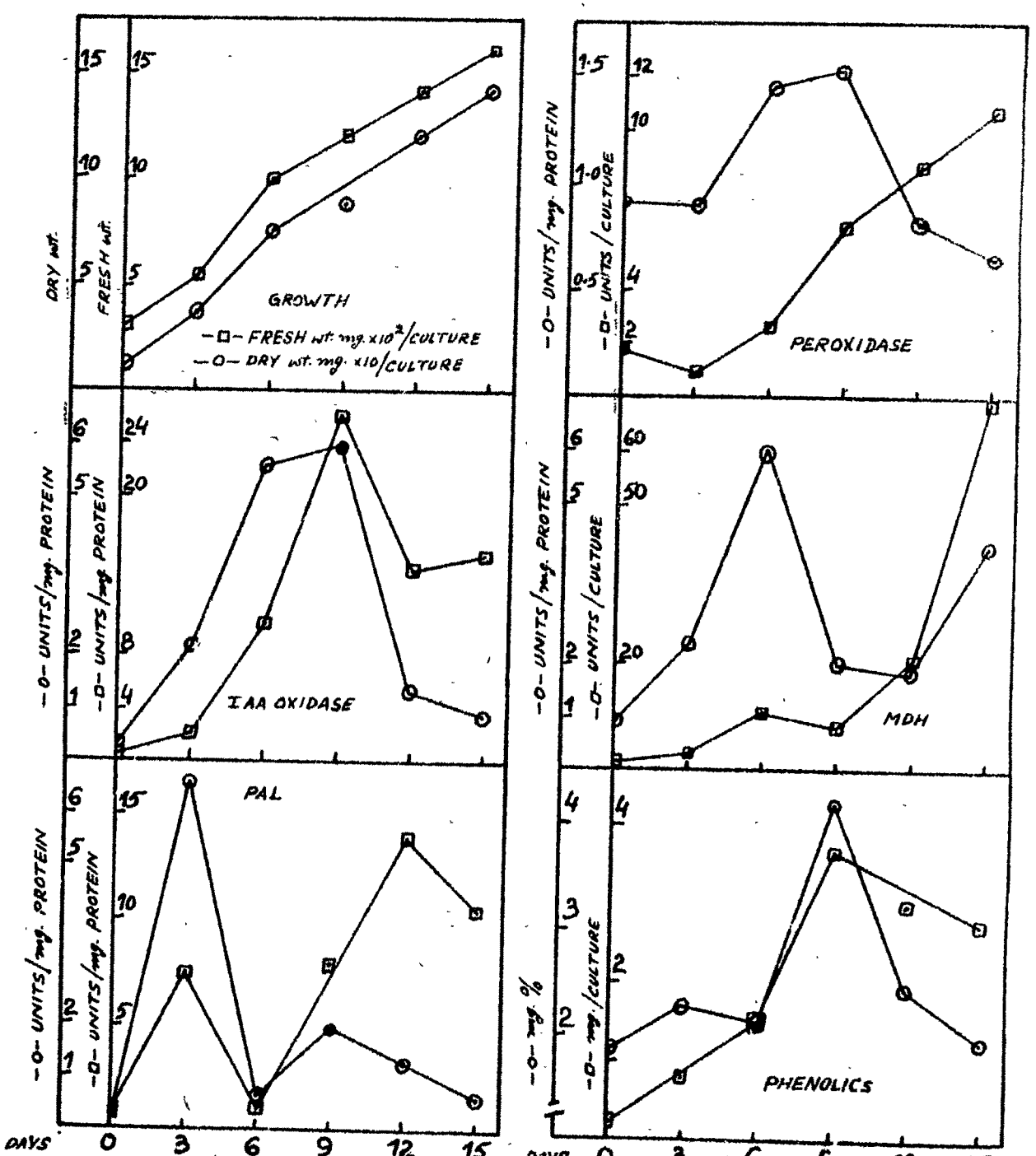
Incubation : 15 days at 26±2° in continuous light

Inoculum : 300±30 mg fresh tissue

Day	Fresh weight mg/cult.	Dry weight mg/cult.	PEROXIDASE		IAA OXIDASE		MDH		PAL		PHENOLICS	
			units/ cult.	units/mg protein	units/ cult.	units/mg protein	units/ cult.	units/mg protein	units/ cult.	units/mg protein	mg/ cult.	mg%
0	300 (±30)	12 (±3)	1.77	0.92	0.57	0.30	1.69	0.88	0.48	0.25	0.22	1.87
3	566.3 (±36.5)	36.7 (±3.6)	1.01	0.90	2.47	2.18	2.71	2.40	7.54	6.66	0.83	2.28
6	1002.6 (±81.2)	73.1 (±4.3)	2.72	1.45	10.39	5.56	11.22	6.01	1.11	0.59	1.54	2.12
9	1208.7 (±79.6)	88.2 (±5.2)	6.43	1.54	25.16	6.02	8.70	2.08	8.04	1.92	3.66	4.16
12	1391.3 (±81.7)	122.8 (±9.7)	8.70	0.81	14.43	1.35	20.86	1.96	14.03	1.32	2.98	2.43
15	1600.2 (±92.9)	141.2 (±11.4)	10.74	0.64	15.56	0.93	69.97	4.20	10.51	0.63	2.76	1.96

Figures in parenthesis represent standard error.

Data represented is average of 5 replicates.



Experimental Details as in Table 52.

Fig. 114.

Table: 53. Growth, Polyphenols and progressive changes in the Activity of Peroxidase, IAA Oxidase, MDH and PAL in diploid callus tissues of N. tabacum L. var. Anand-2 cultured on shoot differentiating medium supplemented with 100 uM ferulic acid.

Medium : MS + 0.3 mg/l IAA + 100 uM ferulic acid + 3% sucrose

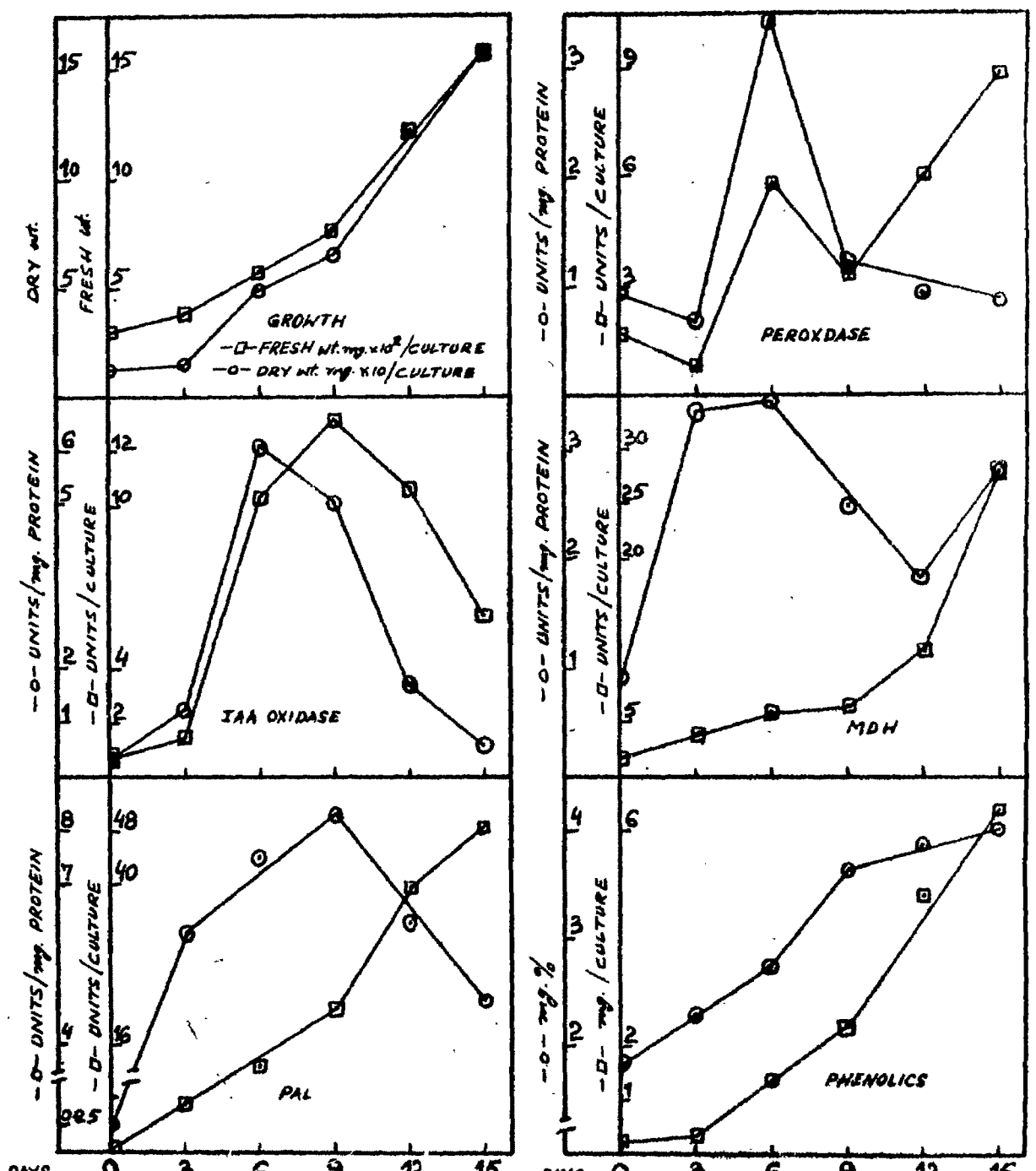
Incubation : 15 days at 26±2° in continuous light

Inoculum : 300±30 mg fresh tissue

Day	Fresh weight mg/cult.	Dry weight mg/cult.	PEROXIDASE		IAA OXIDASE		MDH		PAL		PHENOLICS	
			units/ cult.	units/mg protein	units/ cult.	units/mg protein	units/ cult.	units/mg protein	units/ cult.	units/mg protein	mg/ cult.	mg%
0	300 (±30)	12 (±3)	1.77	0.92	0.57	0.30	1.69	0.88	0.48	0.25	0.22	1.87
3	368.3 (±29.6)	14.5 (±1.3)	0.83	0.69	1.43	1.19	4.12	3.42	7.36	6.11	0.33	2.28
6	579.3 (±42.7)	50.9 (±3.9)	5.91	3.47	10.38	6.10	6.14	3.60	12.81	7.53	1.40	2.76
9	756.1 (±51.5)	66.5 (±4.6)	3.30	1.26	13.36	5.11	6.65	2.54	21.83	8.36	2.40	3.62
12	1229.7 (±66.9)	124.7 (±6.2)	6.07	0.95	10.72	1.68	11.87	1.86	40.27	6.32	4.82	3.87
15	1572.1 (±71.5)	159.4 (±11.5)	8.88	0.89	6.42	0.64	27.82	2.79	49.09	4.93	6.40	4.02

Figures in parenthesis represent standard error.

Data represented is average of 5 replicates.



Experimental Details as in Table 53.

FIG. 115

Table: 54. Growth, Polyphenols and progressive changes in the Activity of Peroxidase, IAA Oxidase, MDH and PAL in diploid callus tissues of N. tabacum L. var. Anand-2 cultured on shoot differentiating medium supplemented with 500 uM ferulic acid.

Medium : MS + 0.3 mg/l IAA + 500 uM ferulic acid + 3% sucrose

Incubation : 15 days at 26±2° in continuous light

Inoculum : 300±30 mg fresh tissue

Day	Fresh weight mg/cult.	Dry weight mg/cult.	PEROXIDASE		IAA OXIDASE		MDH		PAL		PHENOLICS	
			units/ cult.	units/mg protein	units/ cult.	units/mg protein	units/ cult.	units/mg protein	units/ cult.	units/mg protein	mg/ cult.	mg%
0	300 (±30)	12 (±3)	1.77	0.92	0.57	0.30	1.69	0.88	0.48	0.25	0.22	1.87
3	412.9 (±41.6)	16.9 (±1.2)	2.75	1.20	0.15	0.06	5.94	2.59	15.59	6.81	0.54	3.24
6	528.7 (±29.8)	47.9 (±4.3)	3.21	1.47	7.66	3.51	8.61	3.95	9.98	4.57	1.45	3.03
9	665.7 (±51.7)	60.4 (±4.8)	3.72	2.41	14.67	9.49	4.12	2.67	7.39	4.79	1.72	2.86
12	1002.0 (±66.2)	96.5 (±6.6)	4.36	0.64	2.12	0.31	14.07	2.07	38.43	5.66	3.68	3.82
15	1309.0 (±65.9)	126.0 (±7.2)	2.12	0.20	10.27	0.98	38.99	3.74	41.90	4.02	5.12	4.07

Figures in parenthesis represent standard error.

Data represented is average of 5 replicates.

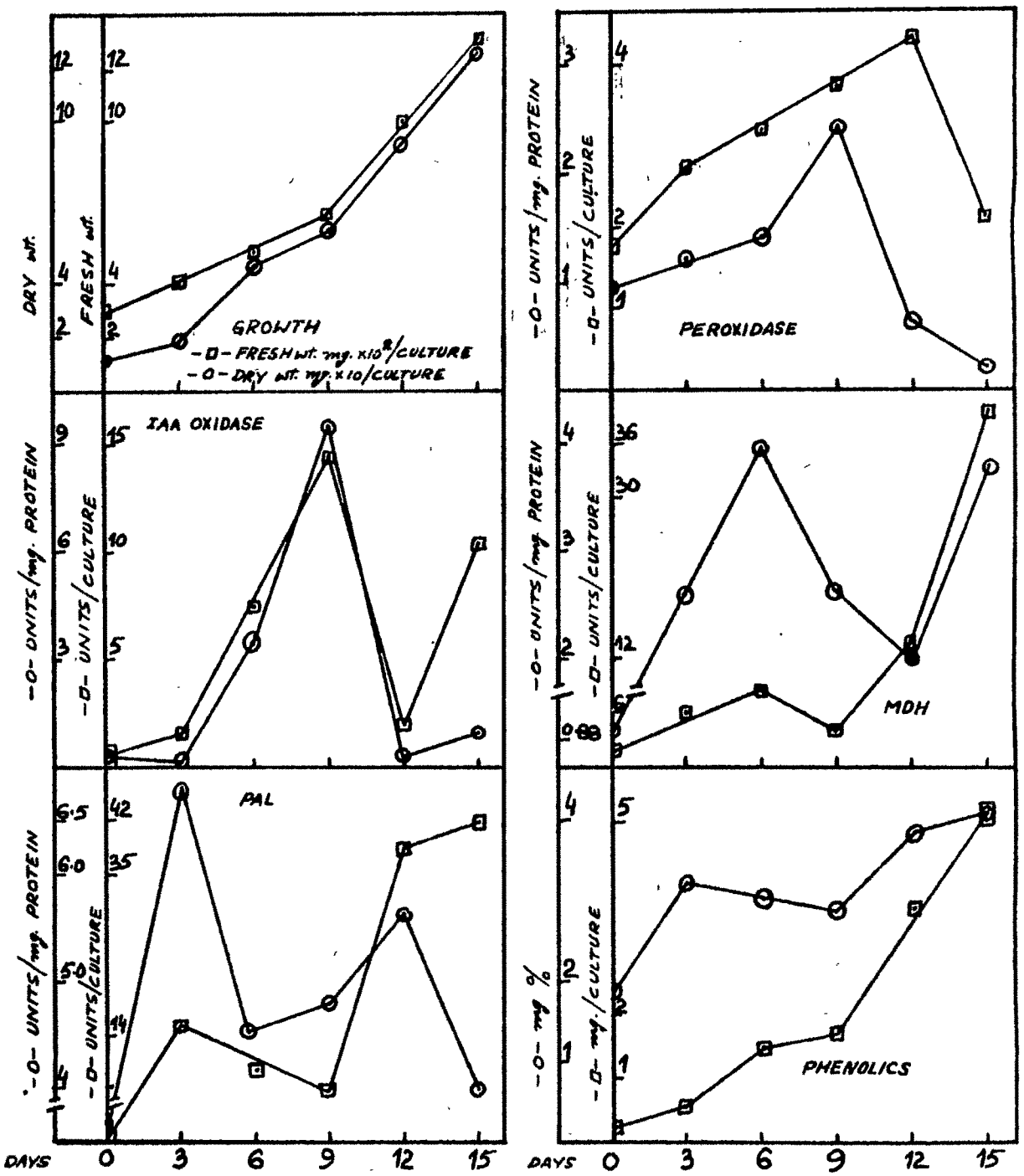


FIG. 116.

Experimental Details as in Table 54.

Table: 55. Growth, Polyphenols and progressive changes in the Activity of Peroxidase, IAA Oxidase, MDH and PAL in diploid callus tissues of N. tabacum L. var. Anand-2 cultured on shoot differentiating medium supplemented with 1.0 uM p-hydroxybenzoic acid.

Medium : MS + 0.3 mg/l IAA + 1.0 uM p-hydroxybenzoic acid + 3% sucrose

Incubation : 15 days at 26±2° in continuous light

Inoculum : 300±30 mg fresh tissue

Day	Fresh weight mg/cult.	Dry weight mg/cult.	PEROXIDASE		IAA OXIDASE		MDH		PAL		PHENOLICS	
			units/ cult.	units/mg protein	units/ cult.	units/mg protein	units/ cult.	units/mg protein	units/ cult.	units/mg protein	mg/ cult.	mg%
0	300 (±30)	12 (±3)	1.77	0.92	0.57	0.30	1.69	0.88	0.48	0.25	0.22	1.87
3	413.8 (±22.1)	23.8 (±2.4)	1.27	0.73	1.11	0.64	7.28	4.20	32.18	18.59	0.65	2.76
6	670.7 (±42.7)	55.6 (±5.3)	2.33	0.91	5.91	2.32	8.45	3.32	27.12	10.66	1.79	3.22
9	976.0 (±49.6)	81.0 (±6.0)	6.29	1.24	22.20	4.39	4.68	0.92	13.01	2.57	2.93	3.62
12	1201.4 (±51.0)	102.5 (±8.2)	4.90	1.47	7.26	2.18	2.33	0.70	10.83	3.25	2.89	2.82
15	1309.9 (±66.9)	111.7 (±10.2)	4.75	2.46	1.79	0.92	1.10	0.57	7.10	3.68	2.09	1.88

Figures in parenthesis represent standard error.

Data represented is average of 5 replicates.

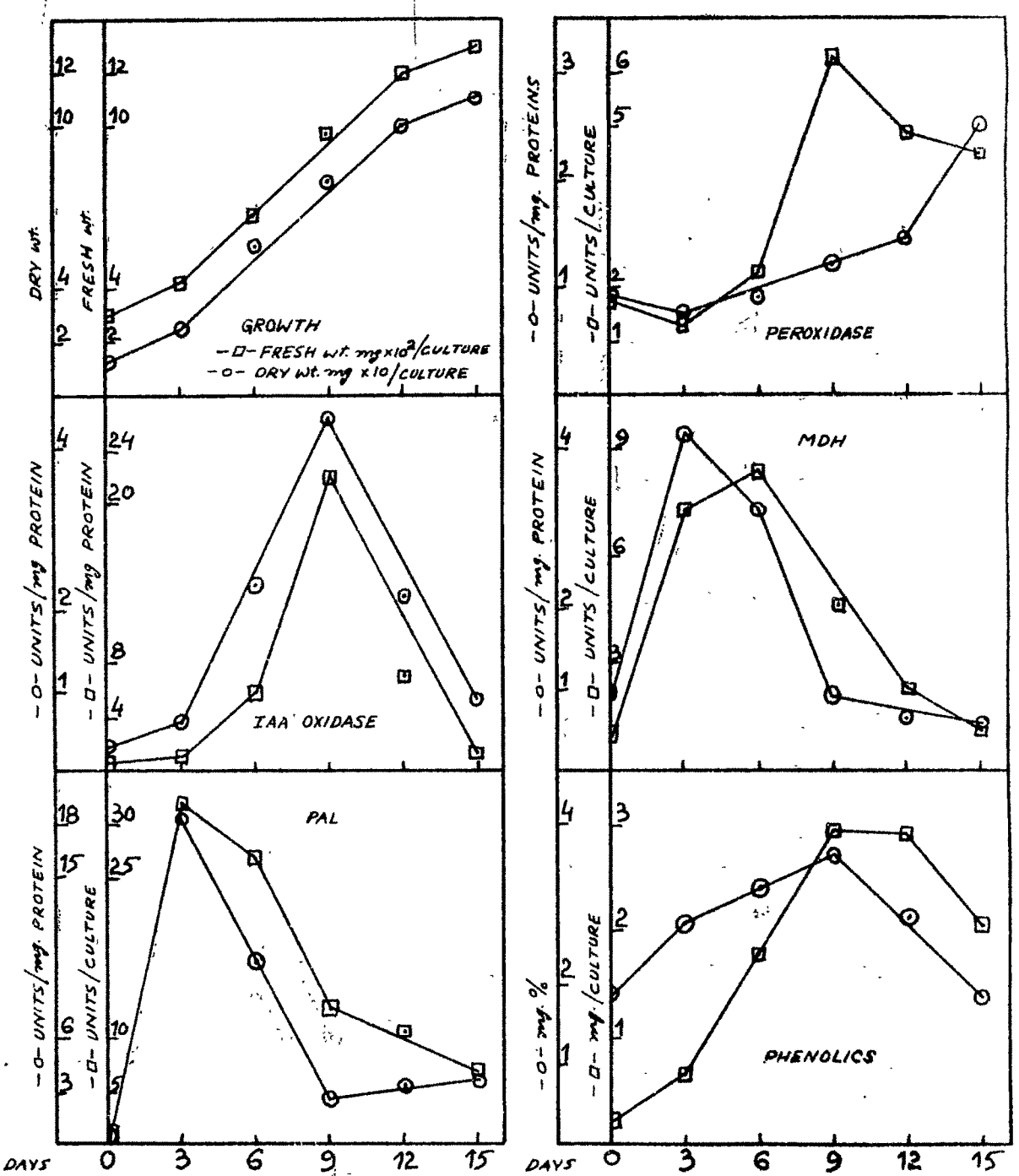


FIG. 117.

Experimental Details as in Table 55.

Table: 56. Growth, Polyphenols and progressive changes in the Activity of Peroxidase, IAA Oxidase, MDH and PAL in diploid callus tissues of N. tabacum L. var. Anand-2 cultured on shoot differentiating medium supplemented with 10.0 uM p-hydroxybenzoic acid.

Medium : MS + 0.3 mg/l IAA + 10.0 uM p-hydroxybenzoic acid + 3% sucrose

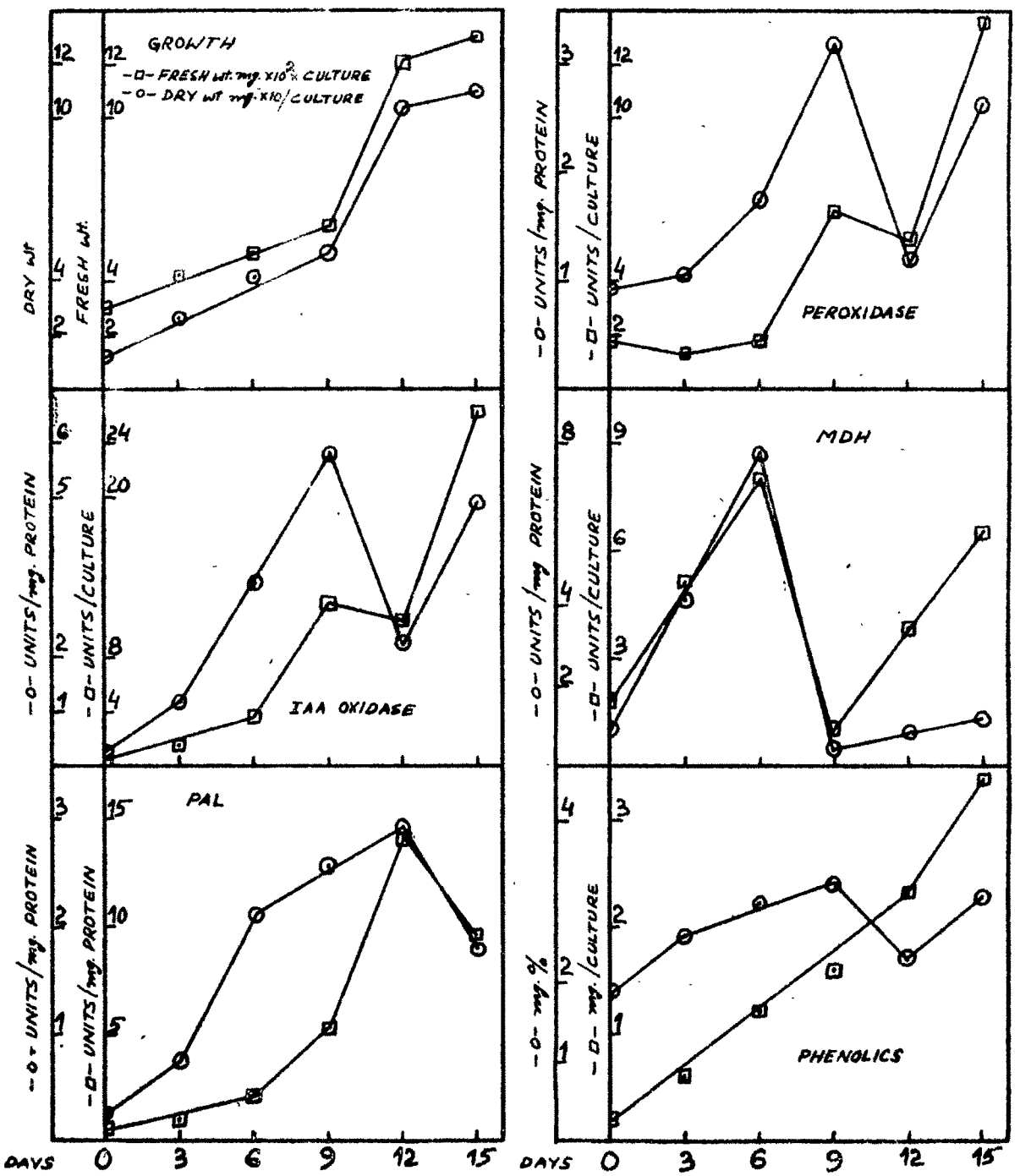
Incubation : 15 days at 26±2° in continuous light

Inoculum : 300±30 mg fresh tissue

Day	Fresh weight mg/cult.	Dry weight mg/cult.	PEROXIDASE		IAA OXIDASE		MDH		PAL		PHENOLICS	
			units/ cult.	units/mg protein	units/ cult.	units/mg protein	units/ cult.	units/mg protein	units/ cult.	units/mg protein	mg/ cult.	mg%
0	300 (±30)	12 (±3)	1.77	0.92	0.57	0.30	1.69	0.88	0.48	0.25	0.22	1.87
3	424.4 (±42.1)	25.7 (±2.0)	1.30	1.05	1.51	1.23	5.09	4.12	0.94	0.76	0.66	2.58
6	493.1 (±36.9)	40.9 (±3.4)	1.78	1.73	3.57	3.46	8.03	7.79	2.18	2.12	1.21	2.96
9	602.2 (±52.0)	50.0 (±5.1)	6.67	3.20	12.16	5.84	0.96	0.46	5.34	2.57	1.61	3.22
12	1238.2 (±61.3)	104.7 (±7.4)	5.52	1.14	10.79	2.23	3.84	0.79	14.18	2.92	2.36	2.26
15	1300.7 (±60.3)	109.9 (±6.9)	13.78	2.63	26.09	4.98	6.46	1.23	9.64	1.84	3.38	3.08

Figures in parenthesis represent standard error.

Data represented is average of 5 replicates.



Experimental Details as in Table 56.

FIG. 118

(c) IAA Oxidase :

The progressive changes of IAA Oxidase activity in callus cultured on shoot inducing medium (MS basal + 0.3 mg/l IAA + 3% sucrose) supplemented with various phenolic acids, are illustrated in Figs. 111-118 and Tables 49-56.

The IAA Oxidase activity in callus cultured on medium supplemented with 100 uM t-cinnamic acid (a) increased between days 0 and 9 to reach peak value. Specific IAA Oxidase activity on the same medium attained peak value, however, on day 6 (Fig. 111, Table 49). On the other hand, on 500 uM t-cinnamic acid (b) containing medium the total IAA Oxidase activity as a consequence of incessant increase between days 0 and 12 attained peak value on day 12. The activity decayed rapidly between days 12 and 15. Specific activity of IAA Oxidase also exhibited essentially identical developmental pattern, though with a slight variation. The specific activity increased between days 0 and 9, reaching peak activity on day 9. Thereof, till day 15 the activity decayed sharply and linearly (Fig. 112, Table 50).

The IAA Oxidase activity per unit protein declined between days 12 and 15 with the incorporation of t-cinnamic acid (100, 500.uM) into the shoot inducing medium. Though same pattern was followed by the enzyme activity on the

standard shoot inducing medium during the corresponding period i.e. days 12 to 15, however, the activity on either of the days was very high as compared with activity on the medium supplemented with t-cinnamic acid (100, 500 μM).

The total and specific activity of IAA Oxidase on both 100 (c) and 500 μM (d) caffeic acid media increased to peak activity between days 0 and 9 before declining thereafter till day 15 (Figs. 113, 114; Tables 51, 52).

As in the case of IAA Oxidase activity per unit protein in floral bud callus cultured on standard shoot inducing medium, with the incorporation of caffeic acid (100, 500 μM) into the medium also resulted in decline of activity between days 12 and 15. However, on both the days the enzyme activity on caffeic acid containing medium was very low as compared with the enzyme activity on the standard shoot inducing medium.

Peak value for total IAA Oxidase activity was reached on day 9 in callus cultured on 100 μM (e) ferulic acid medium. Between days 9 and 15 the activity was on decline. On the same medium the specific IAA Oxidase activity reached peak value on day 6, and was on decline thereafter till day 15 (Fig. 115, Table 53). Double peaked pattern was demonstrated by total and specific activity of IAA Oxidase in callus cultured on 500 μM ferulic acid (f) medium. The first peak

value was reached on day 9 and the second on day 15 (Fig. 116, Table 54).

Low level of ferulic acid (100 μM) favoured decay of IAA Oxidase activity per unit protein between days 12 and 15, whereas, during the corresponding period the enzyme activity increased on 500 μM ferulic acid containing medium. However, on both the media, on either days i.e. days 12 and 15, the activity of the enzyme was appreciably low in comparison with the enzyme activity on standard shoot inducing medium.

Associated with sharp increases between days 3 and 9, the total and specific IAA Oxidase activity in the callus cultured on 1.0 μM p-hydroxybenzoic acid (g) medium, the peak values were attained on day 9 in culture. Between days 9 and 15 the enzyme activity declined along a steep gradient (Fig. 117, Table 55). The culture of callus on medium supplemented with 10 μM p-hydroxybenzoic acid (h) induced double peaked developmental pattern in the total and specific IAA Oxidase activity. The first peak value was attained on day 9 and the second on day 15 (Fig. 118, Table 56).

With the incorporation of 1.0 μM p-hydroxybenzoic acid into the medium the IAA Oxidase activity per unit protein decayed between days 12 and 15, thus following the same

pattern as on the standard shoot inducing medium during the corresponding period. However, the activity was much high on the shoot inducing medium than on the medium containing 1.0 μM p-hydroxybenzoic acid. Increase of p-hydroxybenzoic acid to 10.0 μM in the medium reversed the trend of IAA Oxidase activity development. The activity increased sharply between days 12 and 15, and finally on day 15 was about two times as much as on the corresponding day on the standard shoot inducing medium.

(d) MDH

Progressive changes in the activity of MDH in floral bud callus cultured on medium (MS basal + 0.3 mg/l IAA + 3% sucrose) supplemented with phenolic acids are illustrated in Figs. 111-118 and Tables 49-56.

The total and specific MDH activity in the callus on 100 μM t-cinnamic acid (a) exhibited a typical double peaked pattern. The first peak was reached on day 6 and the second on day 15 (Fig. 111, Table 49). The MDH activity in callus cultured on 500 μM t-cinnamic acid (b) increased unabated between days 0 and 15. The specific activity of MDH on the same medium reached peak value on day 6. Between days 6 and 12 the activity declined, but increased slightly between days 12 and 15 (Fig. 112, Table 50).

The MDH activity per unit protein was on increase between days 12 and 15, in floral bud callus cultured on shoot inducing medium supplemented with t-cinnamic acid (100, 500 μM). On day 15, however, the enzyme activity was higher on 100 μM t-cinnamic acid medium when compared with the activity on standard shoot inducing medium. On the other hand, on 500 μM -t-cinnamic acid medium the activity was rather low in comparison with standard shoot inducing medium on the day 15 of culture.

The MDH activity in the callus cultured on 100 μM caffeic acid (c) medium demonstrated double peaked pattern, one each on days 6 and 12. The specific activity of MDH on the same medium, however, attained only one peak value on day 3. Thereof till day 15 the activity declined continuously (Fig. 113, Table 51). On 500 μM caffeic acid (d) medium the MDH activity attained two peaks, a minor one on day 6 and a major one on day 15. The specific activity of MDH also reached two peaks, one each on days 6 and 15 (Fig. 114, Table 52).

With the incorporation of 100 μM caffeic acid into the medium the MDH activity per unit protein was on decline between days 12 and 15, whereas, during the corresponding period of shoot differentiation on the standard shoot inducing medium the enzyme activity increased. The enzyme

activity on 500 μM caffeic acid medium increased very rapidly between days 12 and 15, thus following essentially the same developmental pattern as on the standard shoot inducing medium. However, the rate of increase of enzyme activity was very rapid with the incorporation of 500 μM caffeic acid into the medium. And, moreover, the actual values of activity were also very high.

On 100 μM ferulic acid (e) medium the MDH activity increased slowly upto day 9. It was followed by sharp increase in activity till day 15. The peak value was realized on day 15. The specific activity of MDH on the same medium, however, exhibited two peaks, one each on days 6 and 15 (Fig. 115, Table 53). On 500 μM ferulic acid (f) medium both, total and specific activities of MDH showed double peaked pattern, one each on days 6 and 15 (Fig. 116, Table 54).

As in the case of floral bud callus cultured on standard shoot inducing medium, the MDH activity per unit protein on 100 and 500 μM ferulic acid containing medium also increased during days 12 and 15. However, the enzyme activity was much higher on the ferulic acid containing medium than on the standard shoot inducing medium.

The MDH activity in the callus cultured on medium

containing 1.0 μM p-hydroxybenzoic acid (g) reached peak value on day 6. Thereafter the activity declined till day 15. The specific activity of MDH, however, reached peak value on day 3 and declined thereof till day 15 (Fig. 117, Table 55). On 10.0 μM p-hydroxybenzoic acid (b) medium the total and specific activities of MDH increased till day 6. Thereafter sharp decline in the activities ensued till day 9. The activities, however, picked up and continued to increase till day 15 (Fig. 118, Table 56).

With the incorporation of 1.0 μM p-hydroxybenzoic acid into the shoot inducing medium, the MDH activity per unit protein decayed between days 12 and 15, while on the standard shoot inducing medium the enzyme activity was on increase. Furthermore, on either of the days the enzyme activity was 2 to 3 times lower on the 1.0 μM p-hydroxybenzoic acid containing medium. With the increase of p-hydroxybenzoic acid level to 10.0 μM in the medium the enzyme activity was on increase between days 12 and 15. However, as in the previous case the activity was appreciably lower than on standard shoot inducing medium.

(e) PAL

Progressive changes of PAL activity in floral bud callus during its culture on IAA containing shoot forming medium

supplemented with different phenolic acids are illustrated in Figs. 111-118 and Tables 49-56.

On 100 μM t-cinnamic acid (a) containing medium the PAL activity reached two peak values during the culture period, one each on days 3 and 15. The specific activity of PAL also reached its peak value on day 3. The activity declined considerably till day 12. By day 15 the specific activity of PAL, however, exhibited a modest increase (Fig. 111, Table 49). On 500 μM t-cinnamic acid (b) medium the PAL activity achieved a minor peak value on day 3 and a major one on day 12. The specific activity also attained two peaks, one each on days 3 and 12 (Fig. 112, Table 50).

The PAL activity attained its peak value on day 12 in the callus when cultured on 100 μM caffeic acid (c) medium. The specific activity, however, demonstrated two peaks, one each on days 3 and 12 (Fig. 113, Table 51). On 500 μM caffeic acid (d) medium double peaked pattern was demonstrated by both total and specific activities of PAL. The total PAL activity achieved the two peaks, one each on days 3 and 12, whereas, specific PAL activity reached peak values on days 3 and 9 (Fig. 114, Table 52).

The total PAL activity in the callus on 100 μM ferulic acid (e) medium continued to increase between days 0 and 12,

whereas, the specific activity reached its peak value on day 9 and declined thereafter till day 15 (Fig. 115, Table 53). On 500 μM ferulic acid (f) medium the total PAL activity reached peak values on days 3 and 15. The specific activity, however, reached peak values on days 3 and 12 (Fig. 116, Table 54).

On the culture of callus on 1.0 μM p-hydroxybenzoic acid medium (g) the total and specific activities of PAL reached their peak values on day 3 of culture. The activities declined thereafter (Fig. 117, Table 55). On the other hand, when floral bud callus was cultured on medium supplemented with 10.0 μM p-hydroxybenzoic acid (h) the peak values of total and specific activity were reached on day 12. Between days 12 and 15 the activity declined (Fig. 118, Table 56).

The total PAL activity per unit protein increased between days 12 and 15 (i.e. the days of shoot differentiation) on the standard shoot inducing medium. With the incorporation of phenolic acid - except 100 μM t-cinnamic acid and 1.0 μM p-hydroxybenzoic acid - into the shoot inducing medium the PAL activity declined between days 12 and 15. Moreover, irrespective of the pattern of development of enzyme activity, the actual enzyme activity was higher on both the days (i.e. days 12 and 15) on phenolic acid containing medium, than on the standard shoot inducing medium.

(f) Phenolics :

Progressive changes in the phenolic content of floral bud callus cultured on shoot forming medium supplemented with various phenolic acids are presented in Figs. 111-118 and Tables 49-56.

On 100 μM t-cinnamic acid (a) medium the phenolic content in the callus increased continuously from 0.22 mg/culture on day 0 to 2.76 mg/culture on day 15. On percentage basis the phenolic accumulation reached its first peak value of 2.68 mg% on day 6 and the second peak value of 2.43 mg% on day 15 (Fig. 111, Table 49). On the other hand, on 500 μM t-cinnamic acid (b) containing medium the phenolic content reached its first peak value of 2.61 mg/culture on day 6 and the second peak value of 3.37 mg/culture was attained on day 12. The phenolic accumulation on percentage basis also followed the same pattern. The first peak value of 3.28 mg% was realized on day 6 and the second peak value of 3.74 mg% on day 12 (Fig. 112, Table 50).

On 100 μM caffeic acid (c) containing medium the phenolic accumulation continued till day 12, reaching its peak value of 3.57 mg/culture. On percentage basis too the peak value of 3.46 mg% was reached on day 12 (Fig. 113, Table 51). Phenolic accumulation reached its peak value of

3.66 mg/culture on day 9 in the callus cultured on 500 μM caffeic acid (d) containing medium. On percentage basis the phenolic content increased till day 3 to 2.28 mg%. By day 6 it dropped to 2.12 mg%. The peak value during the culture period was reached on day 9. The phenolic accumulation on that day was 4.16 mg% (Fig. 114, Table 52).

On 100 μM ferulic acid (e) medium the phenolic content in the callus increased from 0.22 mg/culture on day 0 to 6.40 mg/culture on day 15. On percentage basis too the phenolic accumulation increases from 1.87 mg% on day 0 to 4.02 mg% on day 15 (Fig. 115, Table 53). On 500 μM ferulic acid (f) medium the phenolic content in callus was on increase right from day 0 and reached its peak value of 5.12 mg/culture on day 15. On percentage basis the phenolic content increased from 1.87 mg% on day 0 to 3.24 mg% on day 3. By day 9 it, however, declined to 2.86 mg%. By day 15 the phenolic accumulation had increased again to 4.07 mg% (Fig. 116, Table 54).

In flower bud callus tissues cultured on shoot inducing medium supplemented with 1.0 μM p-hydroxybenzoic acid (g) the phenolic accumulation reached its peak value on day 9, on both culture and percentage basis. The respective values were 2.93 mg/culture and 3.62 mg% (Fig. 117, Table 55). On 10.0 μM p-hydroxybenzoic acid (h) medium the peak phenolic accumulation of 3.38 mg/culture was reached on day 15. On

percentage basis the phenolic content increased from 1.87 mg% on day 0 to 3.22 mg% on day 9. By day 12 the phenolic accumulation had gone down to 2.26 mg%. It, however, increased to 3.08 mg% by day 15 (Fig. 118, Table 56).

On standard shoot inducing medium, with the advent of shoot differentiation the phenolic content in the callus tissue dropped from 2.8 mg% to 2.3 mg% between days 12 and 15. With the exception of 500 μM caffeic acid and 1.0 μM p-hydroxybenzoic acid containing medium, where the phenolic content was lower than on the standard medium, all other phenolic acids facilitated higher levels of phenolic accumulation.

(g) Peroxidase Isoenzymes

Progressive changes in the banding pattern of anodic and cathodic isoperoxidases of floral bud callus tissues cultured on IAA containing shoot forming medium supplemented with various phenolic acids are illustrated in Figs. 111a - 118a.

The culture of floral bud callus on shoot inducing medium supplemented with 100 μM t-cinnamic acid (a) resulted in the repression of original anodic isoperoxidases A_2 and A_3. The original anodic isoenzyme of peroxidase A_1 also remained suppressed on days 3 and 6. It, however, appeared from day 9

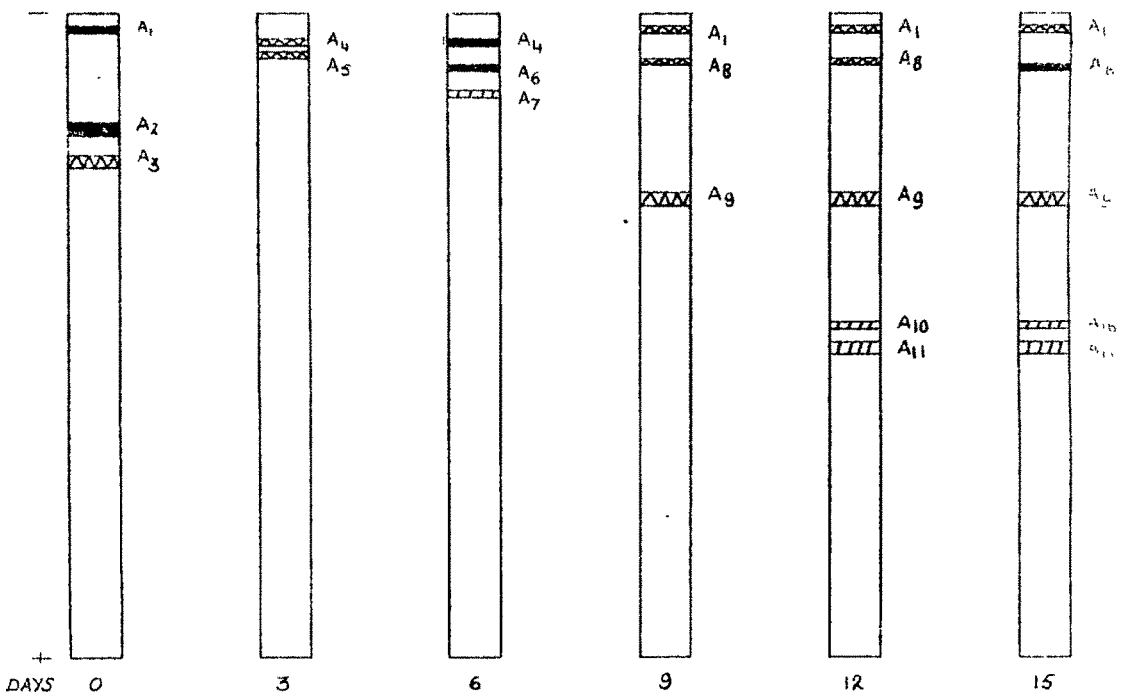
onwards. Slow migrating anodic isoperoxidases A_4 - A_9 . were synthesized at varying time periods of culture. A_4 was synthesized on days 3 and 6, A_5 on day 3, A_6 on days 6 and 15, A_7 on day 6, A_8 on days 9 and 12 and A_9 on days 9, 12 and 15. Two fast migrating isoperoxidases A_{10} and A_{11} appeared on days 12 and 15. Of the three original cathodic isoperoxidases C_1, C_2 and C_3, the isoperoxidase C_2 was repressed right through the culture period. C_1 appeared on days 3 and 15, whereas C_3 was seen only on day 3. Three slow migrating cathodic isoperoxidases C_4 - C_6 were synthesized during the culture period. C_4 was synthesized on all days between days 6-12, C_5 on days 6 and 15 and C_6 on days 9, 12 and 15. No fast migrating cathodic isoperoxidases were synthesized (Fig. 111a).

With the increase of t-cinnamic acid concentration to 500 μM (b) in the medium, the original anodic isoperoxidases A_2 and A_3 were suppressed for the entire duration of culture period. A_1, however, appeared between days 9 and 15. Five slow migrating anodic isoperoxidases A_4 - A_8 were synthesized during the culture period. A_4 was synthesized on all days of culture, A_5 on day 3, A_6 between days 6 - 15, A_7 on day 6 and A_8 on days 9 and 12. One fast migrating anodic isoperoxidase A_9, was synthesized on days 12 and 15. Of the original cathodic isoperoxidases (C_1, C_2 and C_3), C_2 was repressed for the entire culture period. C_1 and C_3, however, appeared

on day 3. Three slow migrating cathodic isoperoxidases C_4 - C_6, were synthesized during the culture period. C_4 was synthesized between days 6 - 15, C_5 on days 9, 12 and 15, and C_6 on days 12 and 15. Like the previous instance, no fast migrating cathodic isoperoxidases were synthesized (Fig. 112a).

Incorporation of 100 μM caffeic acid (c) into the medium resulted in suppression of the three original anodic isoperoxidases A_1 - A_3, right through the culture period. Slow migrating anodic isoperoxidases A_4 - A_{10}, were synthesized at varying periods of culture. A_4 was synthesized on day 3, A_5 between days 6 - 15, A_6 on day 6, A_7 on days 6, 12 and 15, A_8 between days 6 - 15, A_9 between days 9 - 15, and A_{10} on days 12 and 15. Only two of the original cathodic isoperoxidases C_1 and C_3 were retained on day 3 of culture. Thereafter the initial cathodic isoperoxidases were not viewed. Four slow migrating cathodic isoperoxidases C_4 - C_7, appeared at different times during the culture of floral bud callus. C_4 and C_5 were synthesized between days 6 - 15, C_6 between days 9 - 15, and C_7 on days 12 and 15(Fig. 113a). No fast migrating anodic or cathodic isoperoxidases were synthesized.

On 500 μM caffeic acid (d) medium the synthesis of original anodic isoperoxidases A_1 - A_3, was repressed for



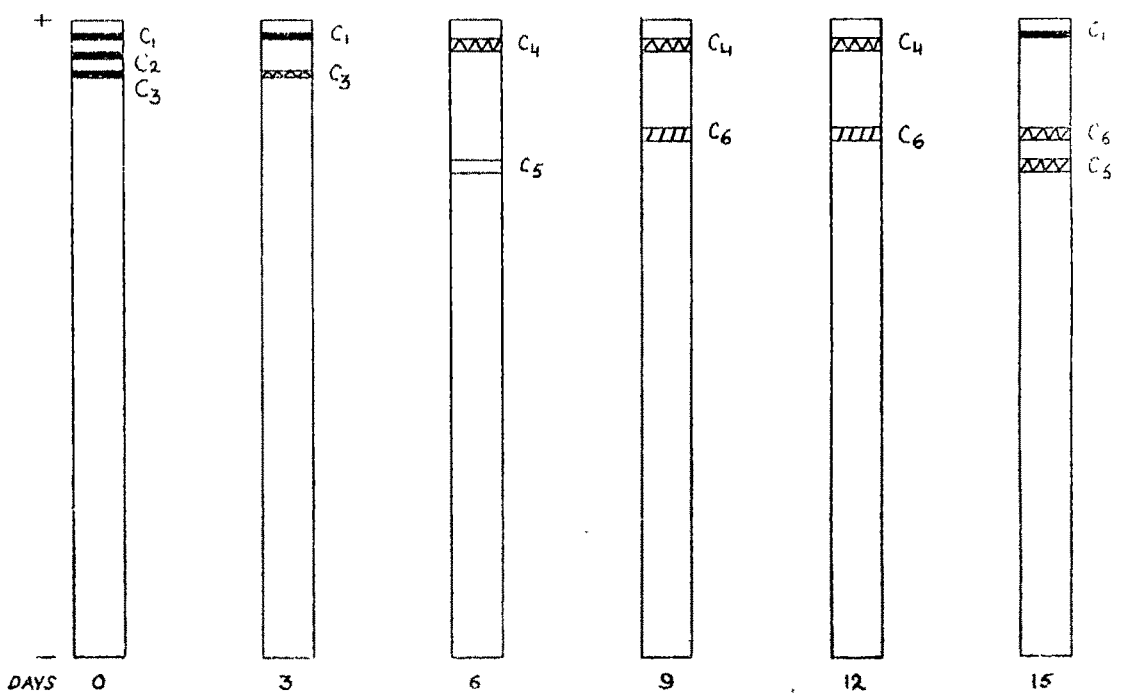
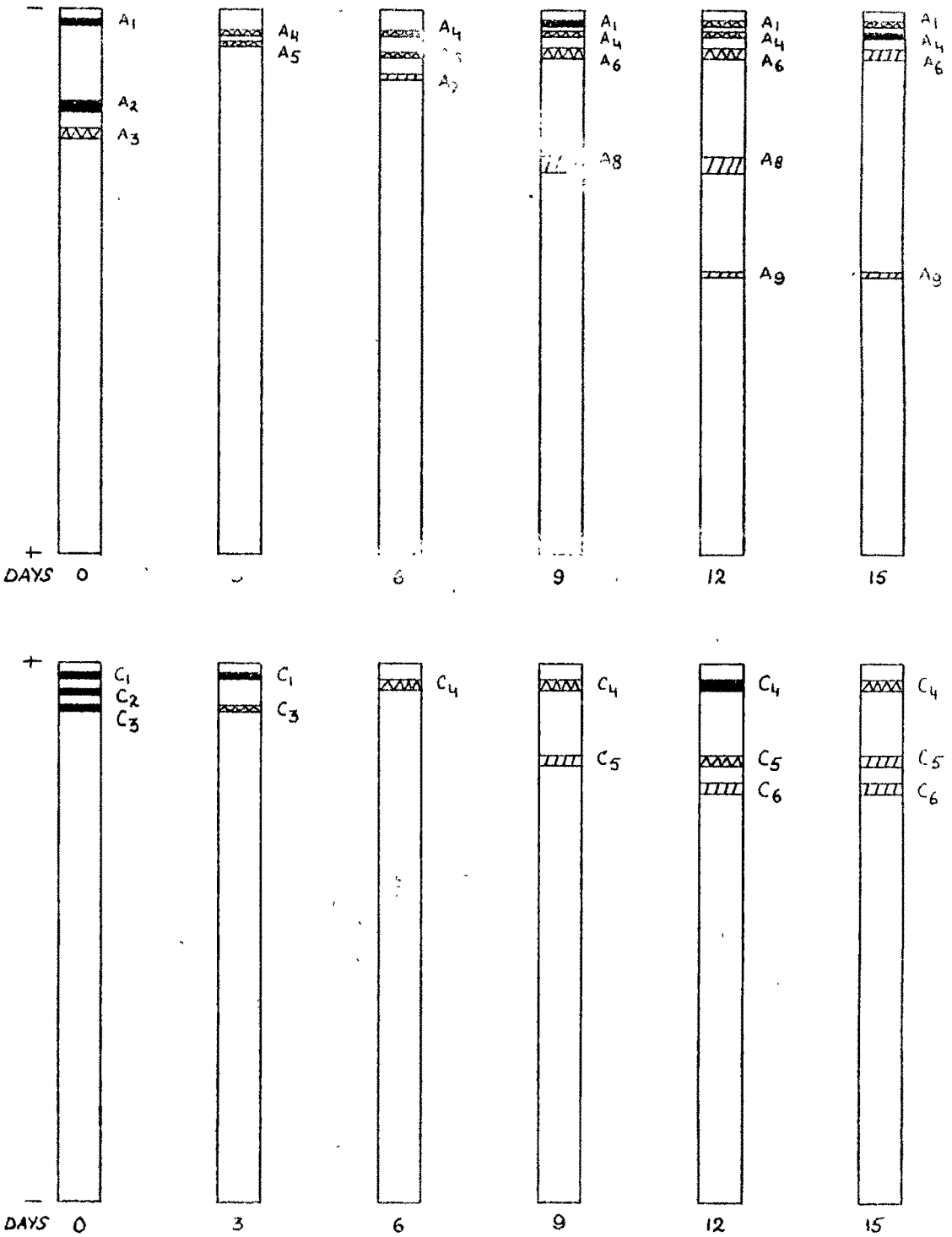


FIG. III a

Anodic and Cathodic Isoperoxidase Patterns

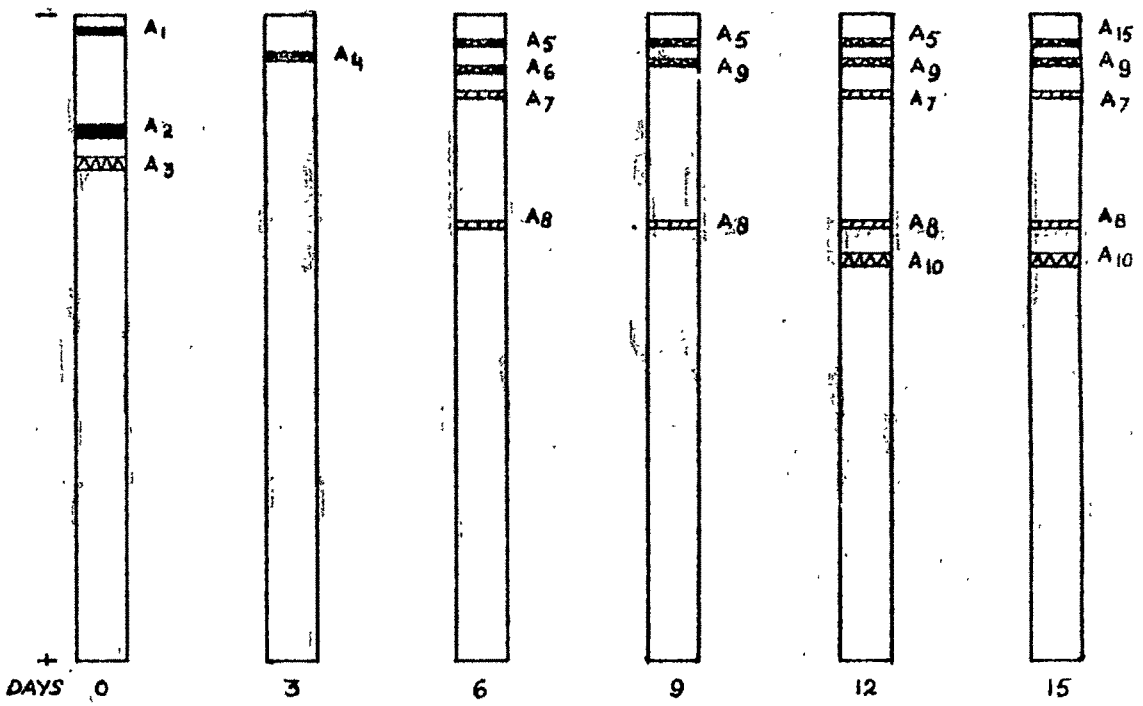
Experimental Details as in Table 49.



Anodic and Cathodic Isoperoxidase Patterns.

Experimental Details as in Table 50.

FIG. 112 a.



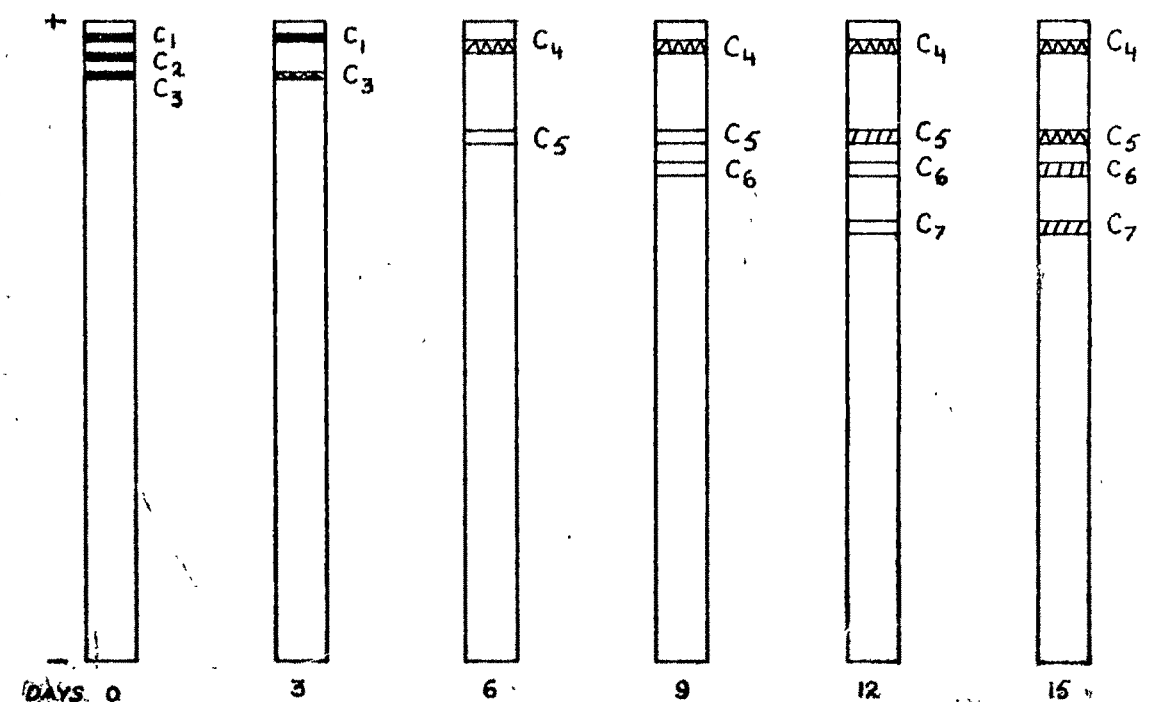


Fig. 113 a.

Anodic and Cathodic Isoperoxidase Patterns:
Experimental Details as in Table 51.

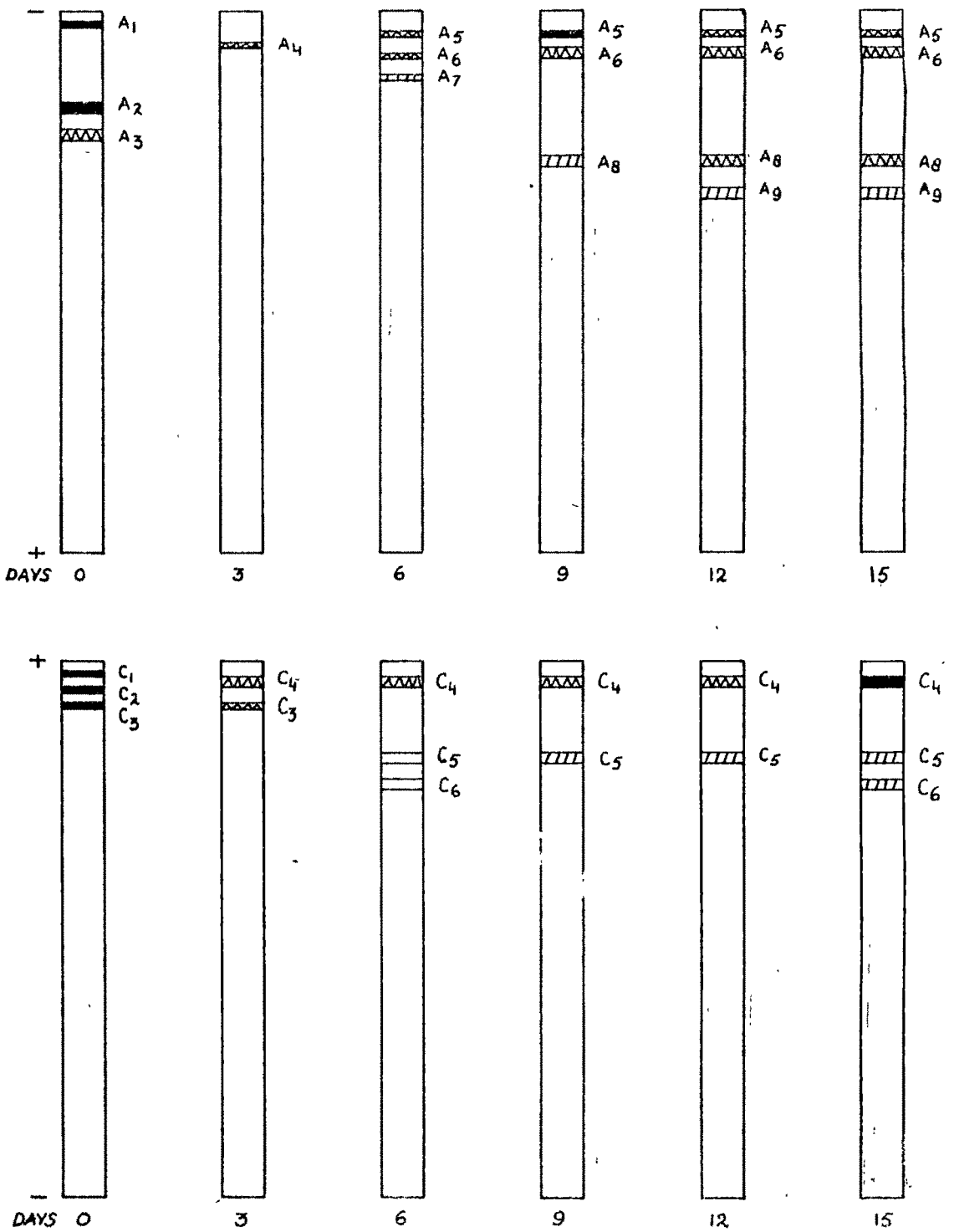
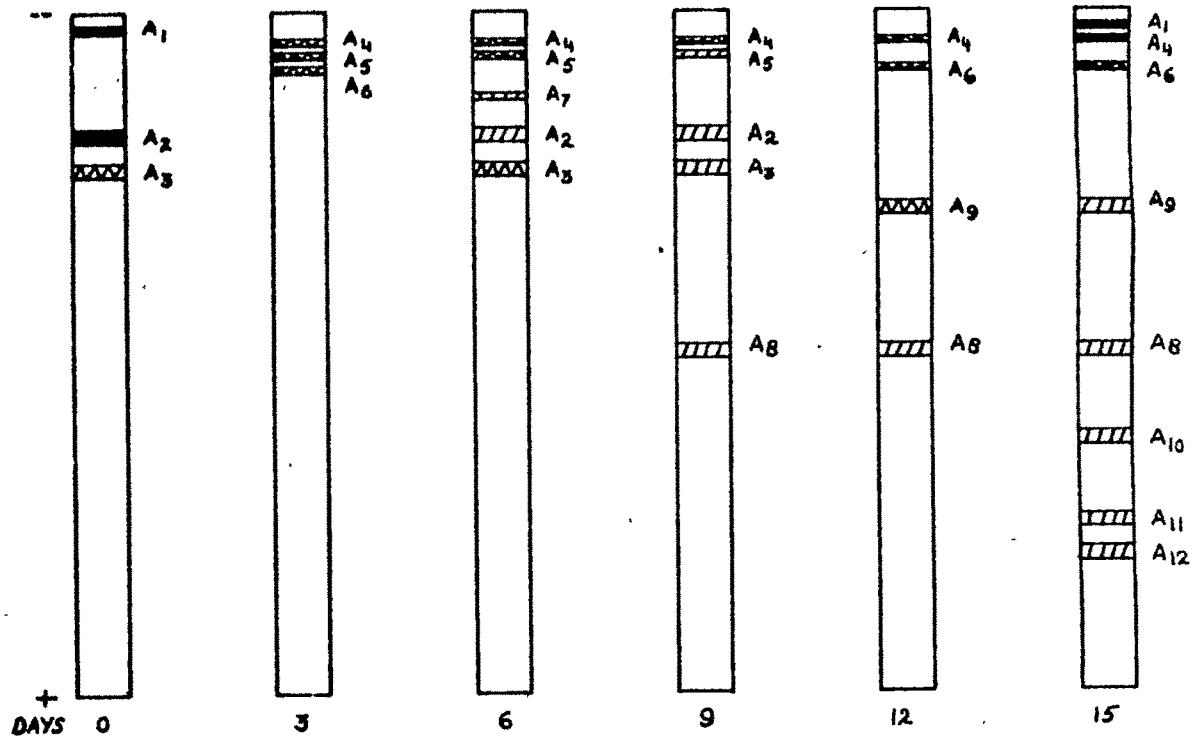


FIG. 114'a

Anodic and Cathodic Isoperoxidase Patterns.
Experimental Details as in Table 52.



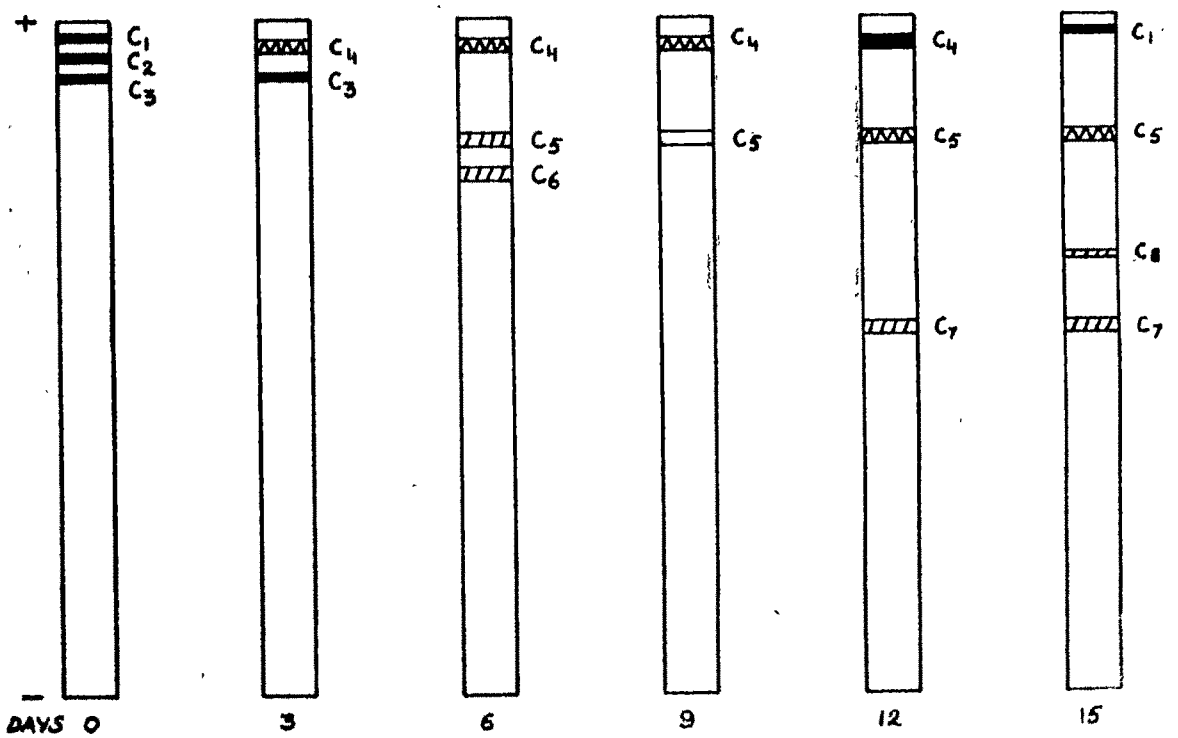
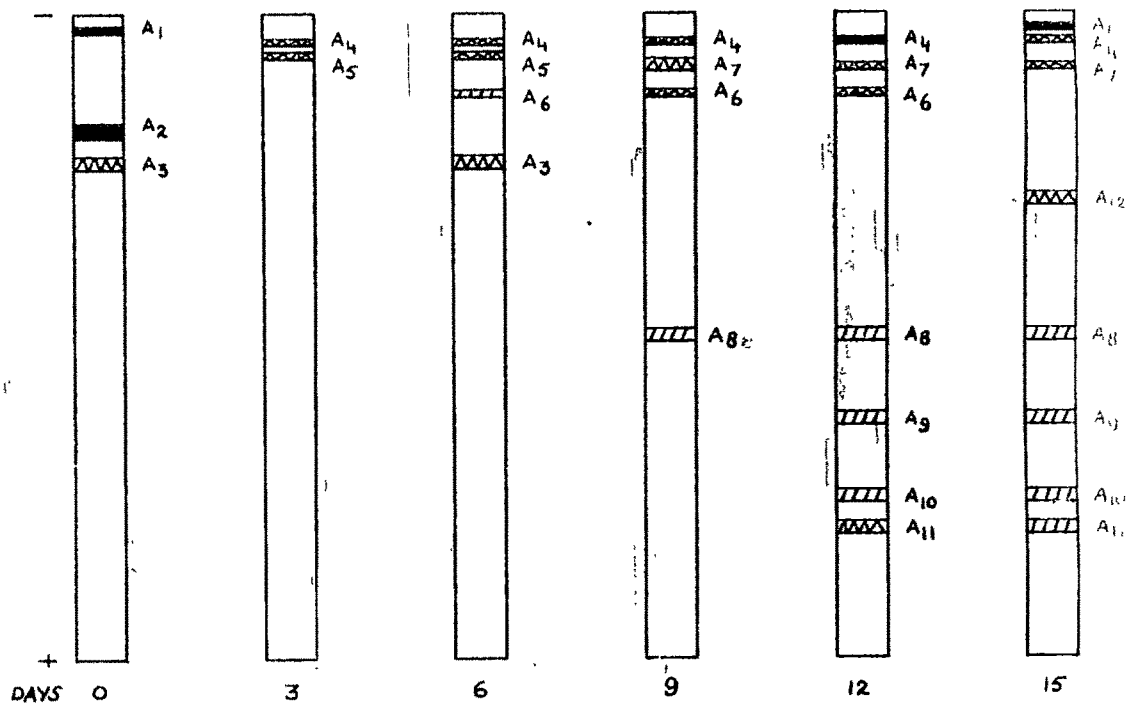


FIG. 115 a

Anodic and Cathodic Isoperoxidase Patterns.
Experimental Details as in Table 53.



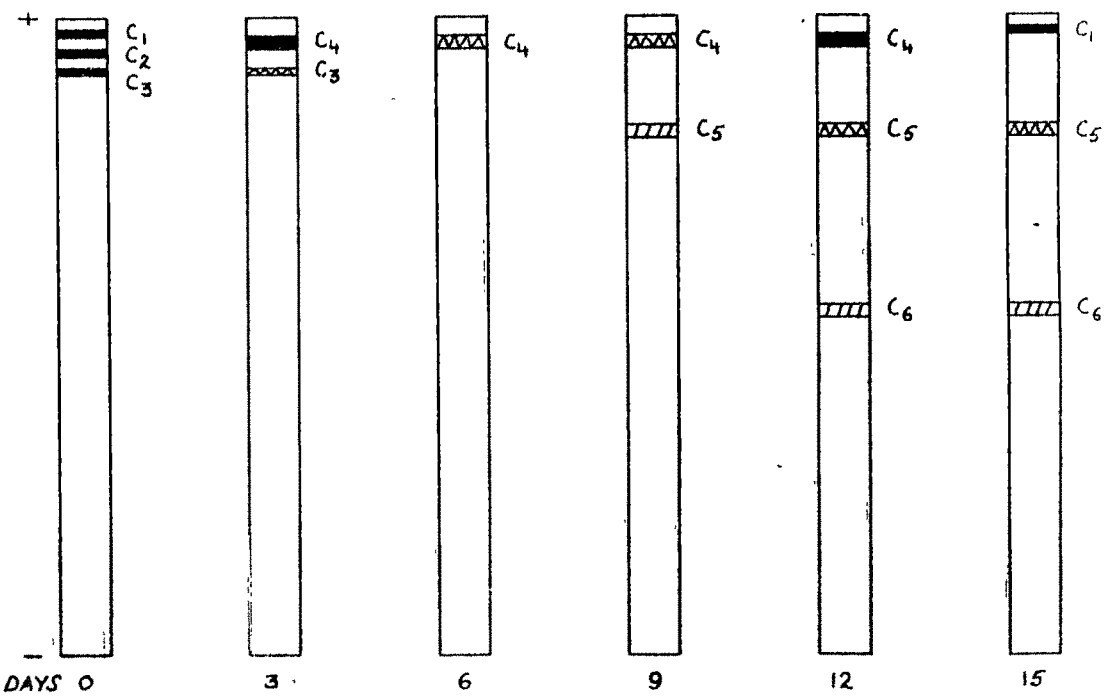


FIG. 116 a.

Anodic and Cathodic Isoperoxidase Patterns

Experimental Details as in Table 54.

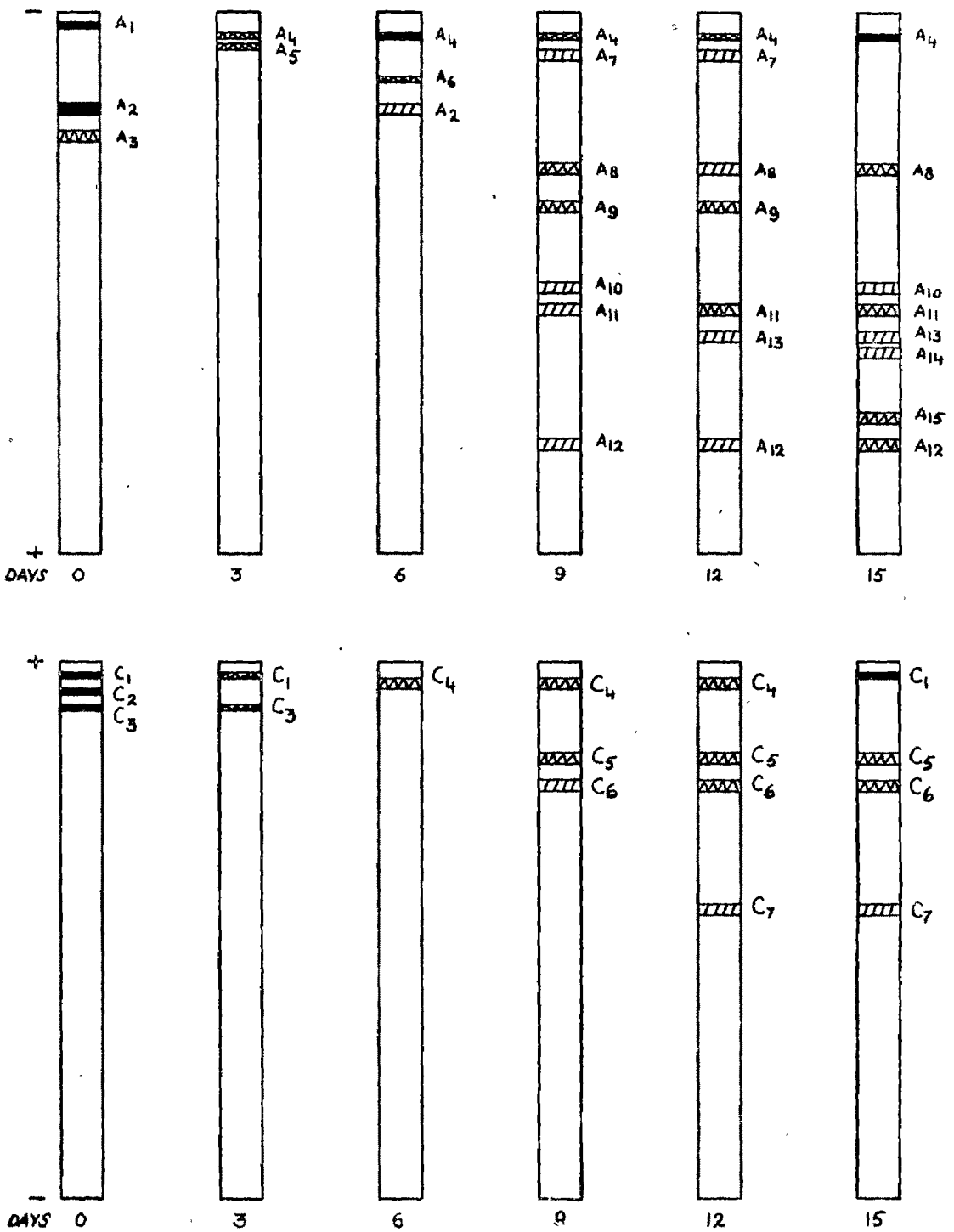
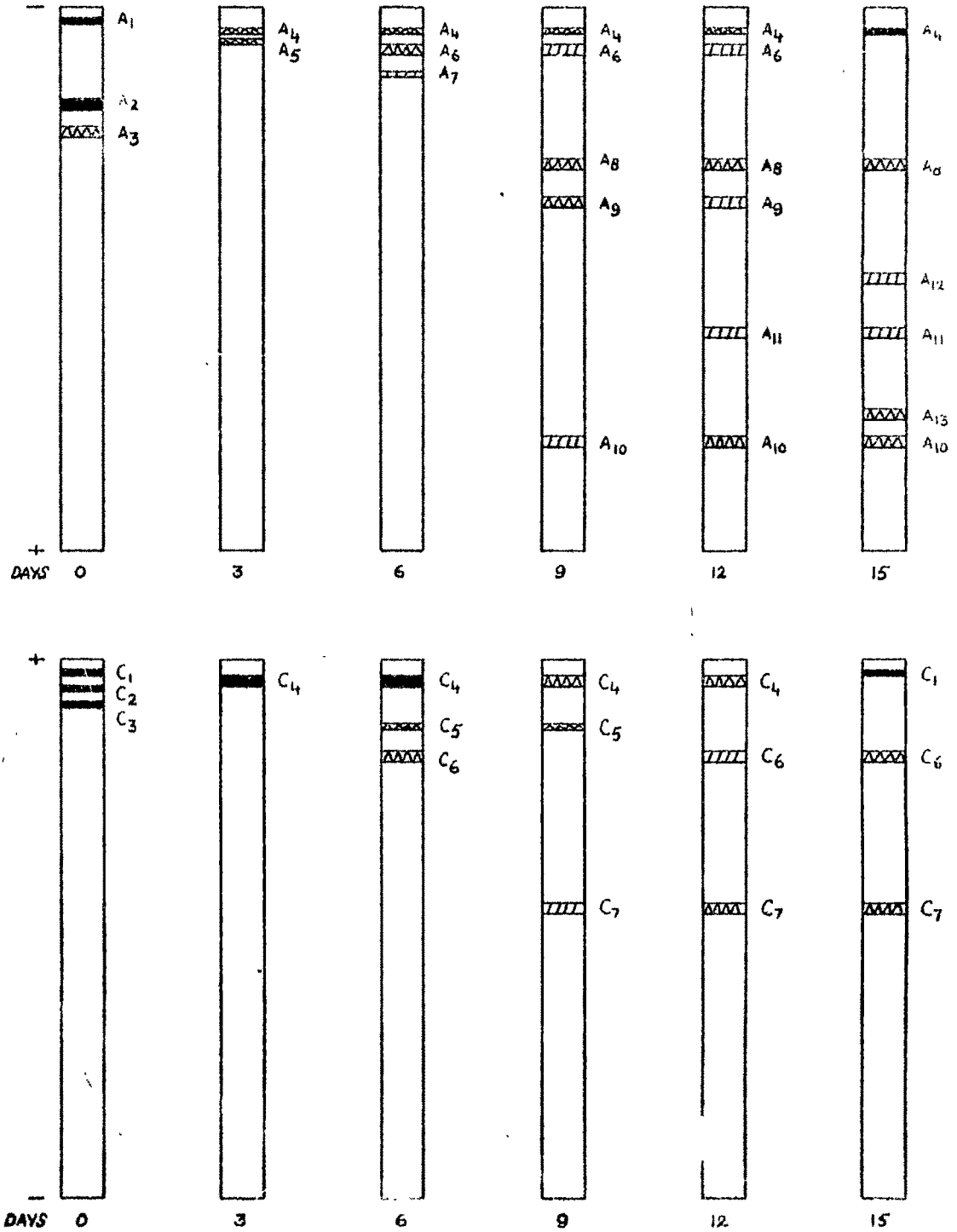


FIG. 117 a

Anodic and Cathodic Isoperoxidase Patterns.
Experimental Details as in Table 55.



Anodic and Cathodic Isoperoxidase Patterns. FIG 11Ba

Experimental Details as in Table 56.

the entire culture period. During the entire culture period only slow migrating anodic isoperoxidases A_4 - A_9, were synthesized. A_4 was synthesized on day 3, A_5 and A_6 between days 6 - 15, A_7 on day 6, A_8 between days 9 - 15 and A_9 on days 12 and 15. Of the three original cathodic isoperoxidases C_1 - C_3, only C_3 appeared on day 3. For the rest of culture period they remained totally repressed. Slow migrating isoperoxidase C_4 was a permanent fixture for the entire culture period of 15 days. Two more slow migrating cathodic isoperoxidases C_5 on days 6 - 15, and C_6 on days 6 and 15 were synthesized (Fig. 114a).

Incorporation of 100 μM ferulic acid (e) into the culture medium resulted in suppression of original anodic isoperoxidase A_1 on all days except day 15. The other two original anodic isoperoxidases A_2 and A_3, were seen only on days 6 and 9. During the culture period slow migrating anodic isoperoxidasea A_4 - A_7 and A_9, were synthesized. A_4 was synthesized between days 3 - 15, A_5 on days 3, 6 and 9, A_6 on days 3, 12 and 15, A_7 on day 6 and A_9 on days 12 and 15. Besides four fast migrating anodic isoperoxidases A_8 and A_{10} - A_{12}, were also synthesized. A_8 appeared on days 9, 12 and 15, and A_{10} - A_{12} on day 15. The original cathodic isoperoxidase C_1 was synthesized only on day 15 and C_3 on day 3. C_2 remained repressed right through the culture

period. Only one fast migrating cathodic isoperoxidase C_7, was synthesized on days 12 and 15. Four slow migrating cathodic isoperoxidases C_4 - C_6 and C_8, were also synthesized at different periods of culture. C_4 was synthesized between days 3 - 12, C_5 between days 6 - 15, C_6 on day 6 and C_8 on day 15(Fig. 115a).

Increasing ferulic acid level to 500 µM (f) in the medium resulted in suppression of original anodic isoperoxidase A_2 for the entire length of culture period. The original anodic isoperoxidase A_1 appeared on day 15 and A_3 on day 6. Four fast migrating anodic isoperoxidases A_8 - A_{11} appeared between days 9 and 15. Five slow migrating ones A_4 - A_7 and A_{12}, were also synthesized. A_4 was synthesized on all days between days 3 - 15, A_5 on days 3 and 6, A_6 between days 6 - 12, A_7 between days 9 - 15 and A_{12} on day 15. The original cathodic isoperoxidases C_1 and C_3 appeared on day 15 and day 3 respectively. C_2 was completely repressed. A fast migrating cathodic isoperoxidase C_6, appeared on days 12 and 15. Two slow migrating cathodic isoperoxidases were also synthesized during culture, C_4 between days 3 - 12 and C_5 between days 9 - 15 (Fig. 116a).

The original anodic isoperoxidases A_1 and A_3, were repressed for the entire tenure of culture period with the incorporation of 1.0 µM p-hydroxybenzoic acid (g) into the medium. A_2, however, appeared on day 6. Slow migrating

anodic isoperoxidases A_4 - A_9, were synthesized at different time period. A_4 appeared on all days between days 3 - 15, A_5 on day 3, A_6 on day 6, A_7 on days 9 and 12, A_8 between days 9 - 15 and A_9 on days 9 and 12. Between days 9 - 15 as many as six fast migrating anodic isoperoxidases A_{10} - A_{15}, were synthesized. A_{10} appeared on days 9 and 15, A_{11} and A_{12} between days 9 - 15, A_{13} on days 12 and 15, and A_{14} and A_{15} on day 15. The original cathodic isoperoxidase C_2 was completely repressed. C_1 appeared on days 3 and 15, and C_3 on day 3. Besides a single fast migrating cathodic isoperoxidase C_7 was synthesized on days 12 and 15. Three slow migrating ones C_4 - C_6, also synthesized. C_4 was synthesized between days 6 - 12 and C_5 and C_6 between days 9 - 15 (Fig. 117a).

Increasing p-hydroxybenzoic acid level in the medium to 10.0 μM (h) resulted in suppression of all the three original anodic isoperoxidases A_1 - A_3, for the entire culture period. Slow migrating anodic isoperoxidases A_4 - A_9, were synthesized at different interval of culture period. A_4 was synthesized on all days between days 3 - 15, A_5 on day 3, A_6 between days 6 - 12, A_7 on day 6, A_8 between days 9 - 15 and A_9 on days 9 and 12 of culture. Between days 9 - 15 four fast migrating anodic isoperoxidases A_{10} - A_{13}, appeared. A_{10} appeared on days 9 - 15, A_{11} on days 12 and 15, and A_{12} and A_{13} on day 15 of culture. The original cathodic

isoperoxidases C_2 and C_3, were completely repressed during the culture period. C_1, however, did appear on day 15. Three slow migrating cathodic isoperoxidases C_4 - C_6, were synthesized at different time intervals. C_4 was synthesized between days 3 - 12, C_5 on days 6 and 9, and C_6 on days 6, 12 and 15. A fast migrating one C_7, appeared between days 9 - 15 (Fig. 118a).

With the incorporation of t-cinnamic acid, caffeic acid, ferulic acid and p-hydroxybenzoic acid into MS basal medium + IAA (0.3 mg/l) + sucrose (3%) - on which differentiation of shoots occurred - the caulogenic response was prevented in its entirity. Even the synthesis of anodic isoperoxidases PS_1, PS_2 and PS_3 which ordinarily preceded shoot differentiation was suppressed.

Expt. 37. Studies with floral bud callus cultured on shoot inducing medium containing ½ X and 2 X Mn^{++} ion concentrations

Healthy callus masses of floral bud callus weighing 300±30 mg by fresh weight were cultured on 20 ml of shoot inducing medium (MS basal + 0.3 mg/l IAA + 3% sucrose). In MS basal medium ½ X and 2 X Mn^{++} ion concentration of the standard level was used. Ordinarily MS basal medium contained 22.3 mg/l $MnSO_4$. Consequently to attain ½ X and 2 X Mn^{++} ion level in the medium, respectively 11.15 mg/l and 44.6 mg/l $MnSO_4$ were added in the basal medium.

The culture vessels were incubated at 26±2° in continuous light for a period of 15 days. Every three days 5 replicates were harvested and analysed for growth, enzymes, peroxidase isoenzyme patterns and phenolic accumulation. The results are presented in Figs. 119, 120 and Tables 57, 58.

(a) Growth :

Growth of floral bud callus cultured on shoot inducing medium containing ½ X and 2 X Mn^{++} ion levels are illustrated in Figs. 119, 120 and Tables 57, 58.

With the use of ½ X Mn^{++} ion level in the medium fresh weight increased 1.67 fold and dry weight 3.36 fold during

the 15 day culture period (Fig. 119, Table 57). On incorporation of 2 X Mn^{++} ion level into the medium, fresh weight increased 1.46 fold and dry weight 3.23 fold during the 15 day culture period (Fig. 120, Table 58).

On shoot inducing medium containing standard Mn^{++} ion level the fresh weight increased 1.64 fold and dry weight 3.63 fold. Hence, on incorporation of $\frac{1}{2}$ X and 2 X Mn^{++} level in the same medium growth on dry weight basis was definitely inhibited. On fresh weight basis growth on $\frac{1}{2}$ X Mn^{++} ion containing medium was comparable to that in the standard medium, but on doubling Mn^{++} ion strength in the medium fresh weight was also less than on standard medium. Furthermore, alteration of Mn^{++} ion level from the standard resulted in complete inhibition of shoot differentiation.

(b) Peroxidase :

Progressive changes of total and specific peroxidase activity in floral bud callus cultured on $\frac{1}{2}$ X and 2 X Mn^{++} ion containing shoot inducing medium are presented in Figs. 119, 120 and Tables 57, 58.

Total peroxidase activity exhibited double peaked pattern of development on $\frac{1}{2}$ X Mn^{++} ion containing medium. The activity decreased till day 3, but then increased to reach first peak value on day 6. The second peak value was

Table: 57. Growth, Polyphenols and progressive changes in the Activity of Peroxidase, IAA Oxidase, MDH and PAL in diploid callus tissues of N. tabacum L. var. Anand-2 cultured on shoot differentiating medium containing ½ X Mn^{++} ion concentration.

Medium : MS (containing ½ X Mn^{++} ion conc.) + 0.3 mg/l IAA + 3% sucrose

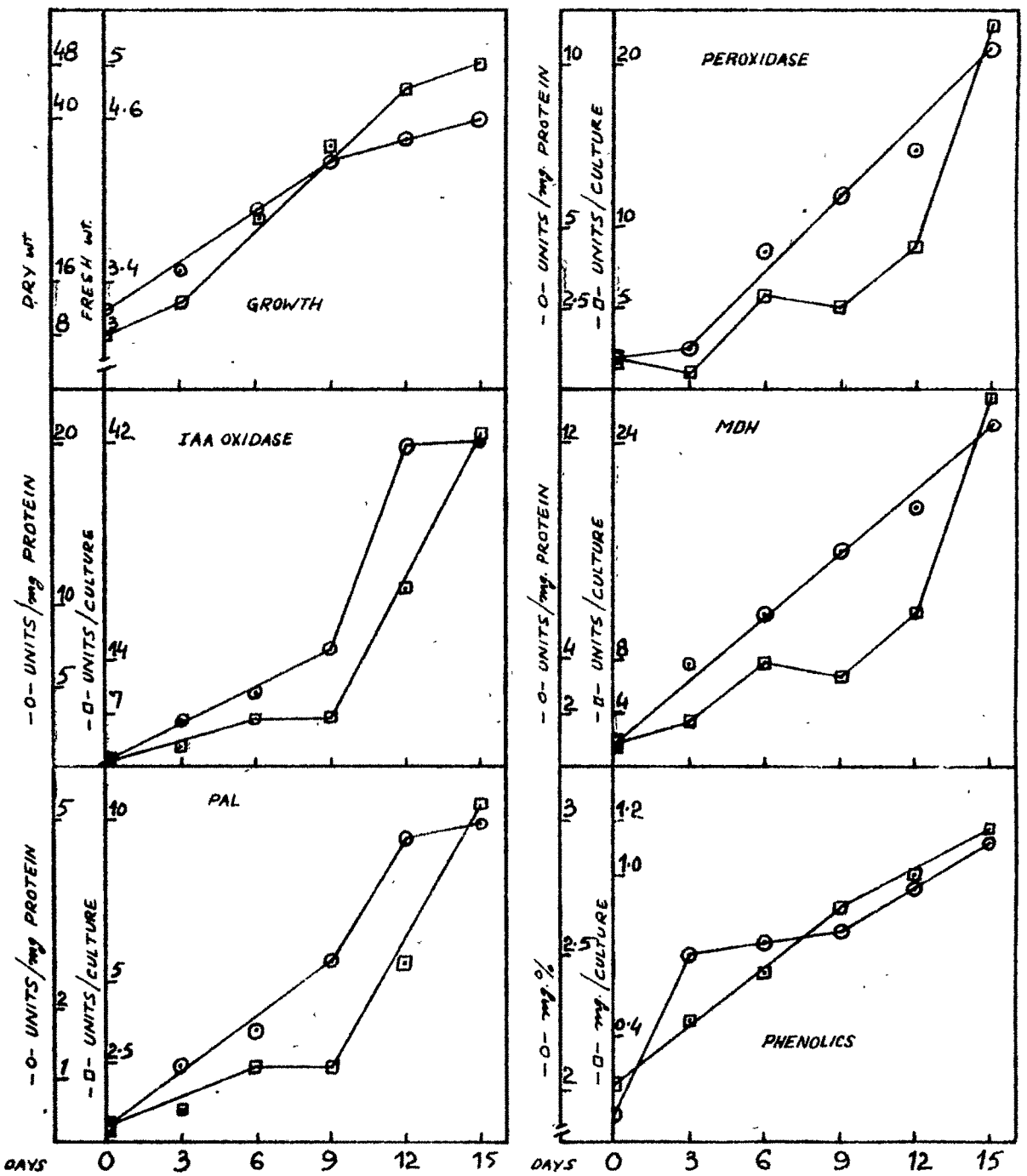
Incubation : 15 days at 26±2° in continuous light

Inoculum : 300±30 mg fresh tissue

Day	Fresh weight mg/cult.	Dry weight mg/cult.	PEROXIDASE		IAA OXIDASE		MDH		PAL		PHENOLICS	
			units/ cult.	units/mg protein	units/ cult.	units/mg protein	units/ cult.	units/mg protein	units/ cult.	units/mg protein	mg/ cult.	mg%
0	300 (±30)	12 (±3)	1.77	0.92	0.57	0.30	1.69	0.88	0.48	0.25	0.22	1.87
3	325.0 (±23.4)	18.61 (±1.6)	1.07	1.24	2.57	2.98	3.35	3.88	1.06	1.23	0.46	2.50
6	388.4 (±33.8)	25.4 (±2.7)	5.88	4.28	6.35	4.62	7.86	5.72	2.42	1.76	0.64	2.54
9	441.6 (±34.1)	33.8 (±3.9)	5.13	5.98	6.33	7.37	6.88	8.02	2.42	2.82	0.87	2.59
12	484.9 (±42.9)	36.8 (±5.8)	8.72	7.37	23.51	19.86	11.39	9.62	5.60	4.73	1.01	2.74
15	502.1 (±40.9)	40.3 (±6.6)	22.59	10.62	43.45	20.42	27.06	12.72	10.53	4.95	1.17	2.92

Figures in parenthesis represent standard error.

Data represented is average of 5 replicates.



Experimental Details as in Table 57.

FIG. 119.

Table: 58. Growth, Polyphenols and progressive changes in the Activity of Peroxidase, IAA Oxidase, MDH and PAL in diploid callus tissues of *N. tabacum* L. var. Anand-2 cultured on shoot differentiating medium containing 2 X Mn^{++} ion concentration.

Medium : MS (containing 2 X Mn^{++} ion conc.) + 0.3 mg/l IAA + 3% sucrose

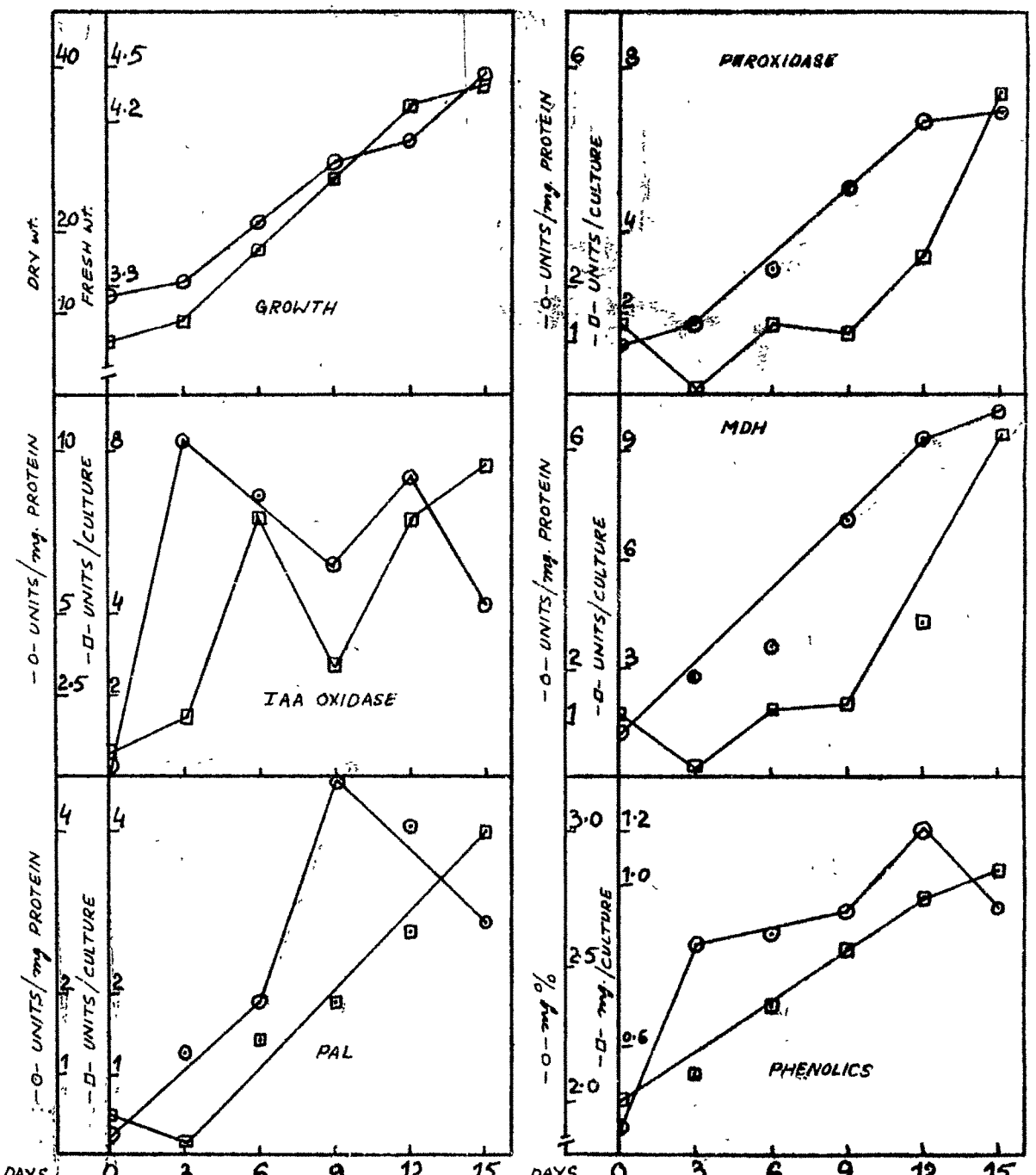
Incubation : 15 days at 26±2° in continuous light

Inoculum : 300±30 mg fresh tissue

Day	Fresh weight mg/cult.	Dry weight mg/cult.	PEROXIDASE		IAA OXIDASE		MDH		PAL		PHENOLICS	
			units/ cult.	units/mg protein	units/ cult.	units/mg protein	units/ cult.	units/mg protein	units/ cult.	units/mg protein	mg/ cult.	mg%
0	300 (±30)	12 (±3)	1.77	0.92	0.57	0.30	1.69	0.88	0.48	0.25	0.22	1.87
3	313.4 (±29.2)	13.9 (±2.3)	0.19	1.36	1.49	10.29	0.27	1.87	0.18	1.26	0.35	2.58
6	350.5 (±30.4)	21.5 (±2.9)	1.76	2.35	6.45	8.62	1.81	2.42	1.43	1.92	0.56	2.62
9	389.8 (±27.6)	28.6 (±2.0)	1.56	3.79	2.71	6.55	1.97	4.76	1.91	4.62	0.77	2.70
12	426.6 (±41.8)	31.5 (±3.6)	3.40	5.01	6.30	9.27	4.27	6.28	2.77	4.08	0.95	3.01
15	438.2 (±38.8)	38.7 (±4.1)	7.36	5.22	7.64	5.42	9.48	6.73	4.03	2.86	1.05	2.72

Figures in parenthesis represent standard error.

Data represented is average of 5 replicates.



Experimental Details as in Table 58.

FIG. 120

reached on day 15. Specific peroxidase activity continued to grow from day 0 and reached peak value on day 15 (Fig. 119, Table 57).

On 2 X Mn^{++} ion containing medium also, the total peroxidase activity declined till day 3. Thereof it increased to reach its first peak value on day 6. The second peak value was attained on day 15. Specific activity was on increase from day 0 till the end of culture period on day 15 (Fig. 120, Table 58).

In contrast with standard shoot inducing medium, the total peroxidase activity per unit protein was on increase right through the culture period when $\frac{1}{2}$ X and 2 X Mn^{++} ion levels were used. On the control medium though the activity was on increase between days 12 - 15 (i.e. days immediately preceding shoot differentiation), it was 6 - 10 times less than on media containing $\frac{1}{2}$ X and 2 X Mn^{++} ion levels.

(c) IAA Oxidase :

Progressive changes of IAA Oxidase activity in floral bud callus cultured on shoot inducing medium containing $\frac{1}{2}$ X and 2 X Mn^{++} ion levels are illustrated in Figs. 119, 120 and Tables 57, 58.

On $\frac{1}{2}$ X Mn^{++} ion containing medium IAA Oxidase activity

increased between days 0 and 6. It struck a pleatu and remained stable between days 6 and 9, thereof it continued to increase rather rapidly till day 15. Specific IAA Oxidase activity was on increase right from the beginning on day 0 till the end of culture period on day 15. The activity developed linearly between days 0 and 9, followed by a sharp rise between days 9 and 12. The activity exhibited only marginal increase between days 12 and 15 (Fig. 119, Table 57).

On 2 X Mn^{++} ion containing medium total IAA Oxidase activity exhibited double peaked pattern of development. The first peak value was reached on day 6. Between days 6 and 9 the activity declined sharply. It, however, increased again between days 9 and 15 to reach the second peak value on day 15. Specific IAA Oxidase activity increased rapidly. between days 0 and 3 to reach its first peak value. The activity decayed linearly between days 3 and 9. Thereof till day 12 the activity increased again reaching its second peak value. Between days 12 and 15 the activity declined rather rapidly (Fig. 120, Table 58).

In contrast with IAA Oxidase activity per unit protein in floral bud callus on the control shoot inducing medium, the activity remained very high when $\frac{1}{2}$ X and 2 X Mn^{++} ion levels were used. The activity was 3 - 7 times more on use

of ½ X and 2 X Mn^{++} ion than on use of standard Mn^{++} ion levels. Furthermore, the patterns of development of IAA Oxidase activity were entirely in contrasts with that on the standard medium.

(d) MDH :

Progressive changes of MDH activity in floral bud callus cultured on shoot inducing medium containing ½ X and 2 X Mn^{++} ion levels, are illustrated in Figs. 119, 120 and Tables 57, 58.

On ½ X Mn^{++} ion containing medium total MDH activity increased between days 0 and 6 to reach its first peak value on day 6. Between days 6 and 9 the activity declined a little, but thereon till day 15 it was on increase. Specific MDH activity, however, exhibited linear increase from day 0 till day 15 (Fig. 119, Table 57).

On 2 X Mn^{++} ion containing medium total MDH activity declined between days 0 and 3. Thereafter it increased continuously to reach its peak value on day 15. Specific MDH activity, on the other hand, was on increase between days 0 and 15, reaching peak value on day 15 (Fig. 120, Table 58).

On the standard shoot inducing medium as well as on the

media containing ½ X and 2 X Mn^{++} ion levels, MDH activity per unit protein was on increase between days 9 and 15 (i.e. the days immediately preceding shoot differentiation on standard shoot inducing medium). The activity increased during that period from 0.99 - 1.66 units/mg protein on standard shoot inducing medium, from 8.02 - 12.72 units/mg protein on ½ X Mn^{++} ion containing medium and from 4.76 - 6.73 units/mg protein on 2 X Mn^{++} ion containing medium. It became apparent that with alteration of Mn^{++} ion level in the shoot inducing medium, the MDH activity was enhanced considerably.

(e) PAL :

Progressive changes of total and specific PAL activity in floral bud callus tissues cultured on shoot inducing medium containing ½ X and 2 X Mn^{++} ion levels, are illustrated in Figs. 119, 120 and Tables 57, 58.

On shoot inducing medium containing ½ X Mn^{++} ion level, total PAL activity was on increase during the first 6 days of culture. Between days 6 and 9, the activity remained stable. Thereof it increased sharply to reach peak value on day 15. The specific PAL activity, on the other hand, was continuously on increase from day 0 till day 15 (Fig. 119, Table 57).

Total PAL activity on medium containing 2 X Mn^{++} ion concentration declined till day 3. Thereafter it increased continuously till day 15. Specific PAL activity increased between days 0 and 9 reaching peak value on day 9. Between days 9 and 15 the activity was on decline (Fig. 120,Table 58).

In contrast with total PAL activity per unit protein in floral bud callus cultured on standard shoot inducing medium, the PAL activity in callus tissues cultured on $\frac{1}{2}$ X and 2 X Mn^{++} ion containing medium remained higher on all days of culture, irrespective of the pattern of development followed.

(f) Phenolics :

The changes of phenolic accumulation in floral bud callus cultured on shoot inducing medium containing $\frac{1}{2}$ X and 2 X Mn^{++} ion levels are illustrated in Figs. 119, 120 and Tables 57, 58.

In floral bud callus cultured on shoot inducing medium containing $\frac{1}{2}$ X Mn^{++} ion level, the phenolic accumulation was on increase from day 0 till day 15. It increased from 0.22 mg/culture on day 0 to 1.17 mg/culture by day 15. On percentage basis also the peak phenolic accumulation was reached on day 15. The phenolic content increased from 1.87 mg% on day 0 to 2.92 mg% on day 15 (Fig. 119, Table 57).

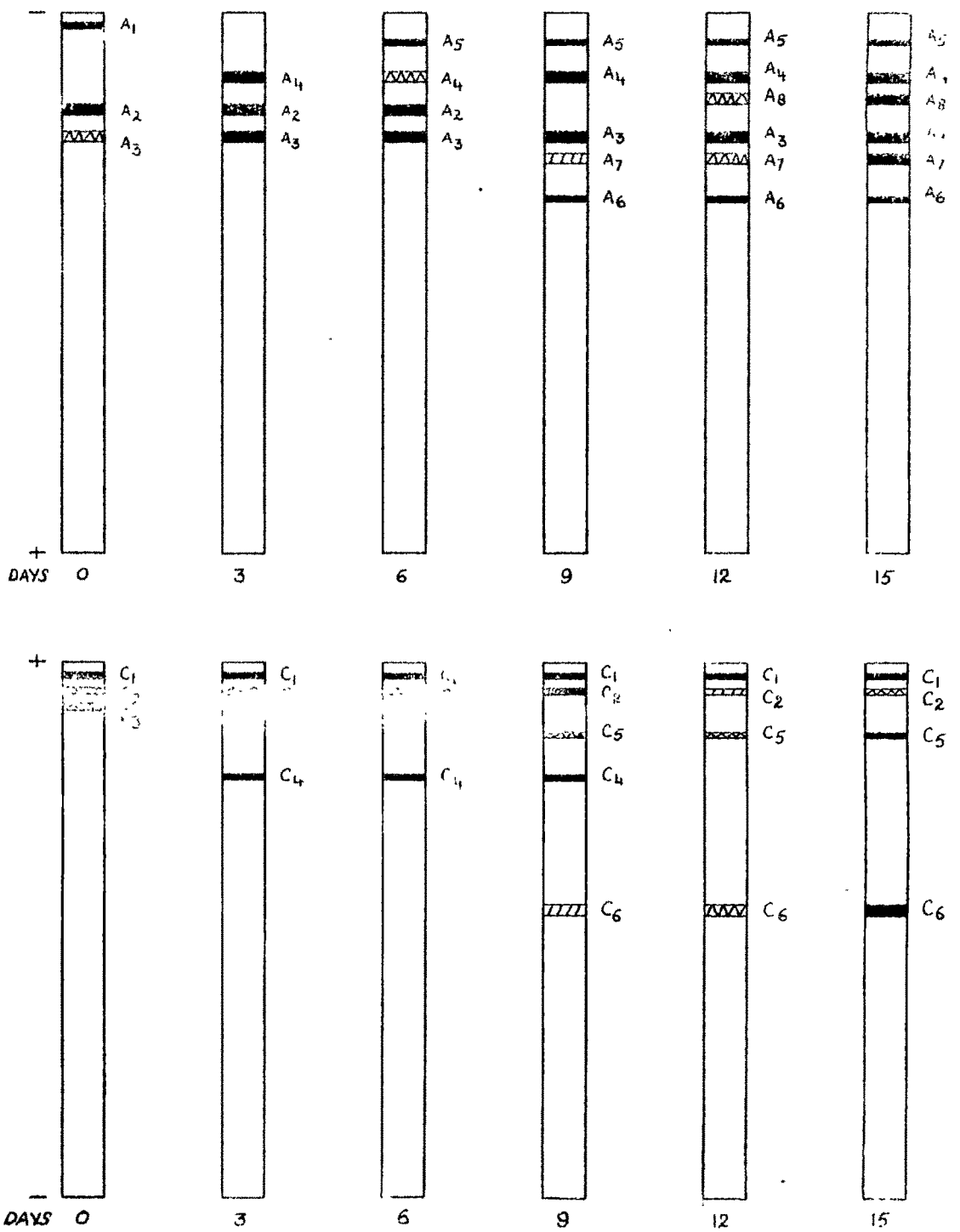
On 2 X Mn^{++} ion containing medium also as in the previous case the phenolics accumulated right through the culture period of 15 days. Phenolic content increased from 0.22 mg/culture to 1.05 mg/culture. On percentage basis peak phenolic accumulation of 3.01 mg% was reached on day 12. Between days 12 and 15 the phenolic content was on decline (Fig. 120, Table 58).

On standard shoot inducing medium the phenolic content declined from 2.8 mg% to 2.3 mg% between days 12 - 15 (i.e. the days immediately preceding shoot differentiation). During the corresponding period phenolic content increased from 2.74 mg% to 2.92 mg% on $\frac{1}{2}$ X Mn^{++} ion containing medium, and declined from 3.01 mg% to 2.72 mg% on 2 X Mn^{++} ion containing medium.

(g) Peroxidase Isoenzymes :

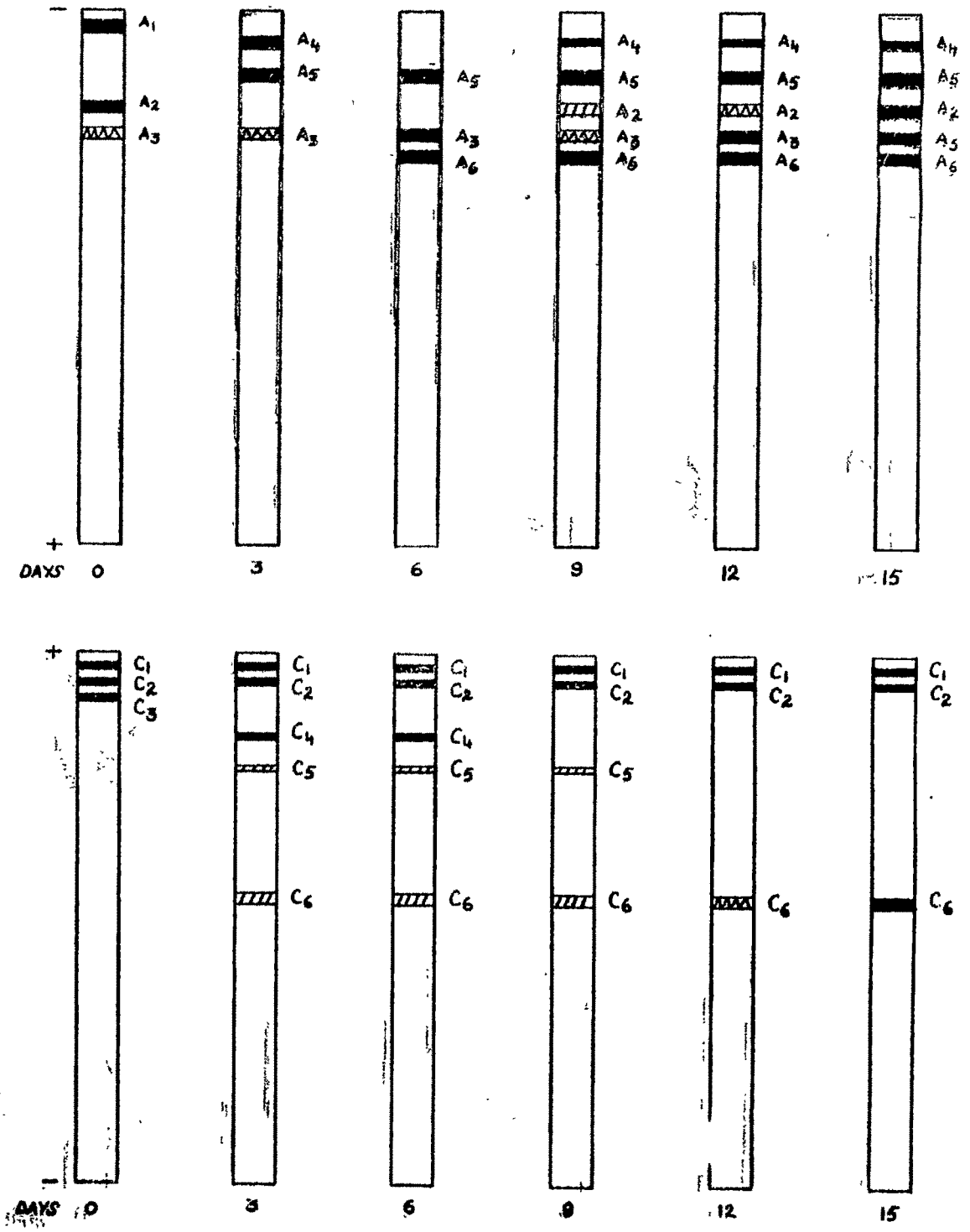
Progressive changes of anodic and cathodic banding patterns of peroxidase isoenzymes in floral bud callus cultured on shoot inducing medium containing $\frac{1}{2}$ X and 2 X Mn^{++} ion levels are illustrated in Figs. 119a and 120a.

On shoot inducing medium containing $\frac{1}{2}$ X Mn^{++} ion level the initial anodic isoperoxidase A_1 remained repressed for the entire length of culture period. Initial anodic



Anodic and Cathodic Esperoxidase Patterns. FIG. 119 a

Experimental Details as in Table 57.



Anodic and Cathodic Isoperoxidase Patterns. FIG. 120 a
Experimental Details as in Table 5B

isoperoxidase A_2 was synthesized on days 3 and 6, and A_3 was synthesized on all days of culture. During the 15 day culture period 5 slow migrating anodic isoperoxidasea A_4 - A_8, were synthesized. A_4 was synthesized between days 3 - 15, A_5 between days 6 - 15, A_6 and A_7 between days 9 - 15 and A_8 on days 12 and 15. On cathodic scale the original isoperoxidase C_3 remained repressed for the entire length of culture period. Other two original ones C_1 and C_2 were synthesized on all days of culture. C_4 a slow migrating cathodic isoperoxidase was synthesized between days 3 - 9. Another slow migrating one C_5 was synthesized between days 9 - 15. A fast migrating cathodic isoperoxidase C_6 was synthesized on days 9, 12 and 15 (Fig. 119a).

On 2 X Mn^{++} ion containing shoot inducing medium the original anodic isoperoxidase A_1 remained suppressed for the whole culture period. On the other hand, other original one, A_2 was synthesized on days 9, 12 and 15. The third original anodic isoperoxidase A_3 was synthesized on all days of culture. Three slow migrating anodic isoperoxidases A_4 - A_6, were synthesized during the culture period. A_4 was synthesized on all days of culture except day 6, A_5 between days 3 - 15, and A_6 between days 6 - 15. On the cathodic scale initial isoperoxidases C_1 and C_2 were synthesized on all days of culture. The third original one C_3 remained repressed for

the entire culture period. Two slow migrating cathodic isoperoxidases C_4 on days 3 and 6, and C_5 on days 3, 6 and 9 were also synthesized. C_6 a fast migrating one was synthesized on all days of culture (Fig. 120a).

On use of $\frac{1}{2}$ X and 2 X Mn^{++} ion level in the shoot inducing medium, shoot differentiation was completely inhibited, also anodic isoperoxidases PS_1, PS_2 and PS_3 were inhibited. In earlier experiment these isoperoxidases were seen to precede shoot differentiation and were also detected in peroxidase preparation of regenerated shoots.

Expt. 38. Studies with haploid tobacco callus tissues cultured on shoot inducing medium

Healthy callus masses of haploid tobacco weighing 300±30 mg by fresh weight were cultured on 20 ml of the shoot differentiating medium. The medium used was : MS (basal) + 0.3 mg/l IAA + 3% sucrose.

The culture vessels were incubated at 26±2° in continuous light for a maximum period of 9 days. Every 3 days, 5 replicates were harvested and analysed for growth, enzymes, isoperoxidase patterns and phenolic accumulation.

(a) Growth :

Growth of haploid tobacco callus cultured on shoot inducing medium and measured as increase in fresh and dry weights are illustrated in Fig. 121 and Table 59.

Growth of haploid tobacco callus tissue increased rapidly on fresh weight basis between days 0 and 3. Thereafter till day 6 the growth slowed down. However, between days 6 and 9 fresh weight increased again at a rapid rate. During the 9 day culture period fresh weight increased 2.77 fold. Growth on dry weight basis exhibited two phases of growth. Between days 0 and 3, dry weight increased rather slowly, thereafter till day 9 it increased rapidly along a linear gradient. Dry weight increased 4.97 fold during the

9 day culture period (Fig. 121, Table 59).

On the above mentioned shoot inducing medium, shoots were differentiated from haploid tobacco callus on day 9 of culture with about 75 per cent frequency.

(b) Peroxidase :

Progressive changes of peroxidase activity in haploid callus cultured on shoot inducing medium are illustrated in Fig. 121, Table 59.

The total peroxidase activity increased right from the beginning and reached its peak value on day 9. The specific activity of peroxidase on the same medium followed a sigmoid pattern of development. Specific peroxidase activity increased slightly between days 0 and 3, followed by a sharp increase between days 3 and 6. The last phase was characterized by slow increase of activity between days 6 and 9 (Fig. 121, Table 59).

The peroxidase activity per unit protein was on the increase, though at a slow rate, during days (6-9) immediately preceding shoot differentiation.

(c) IAA Oxidase :

The progressive changes of IAA Oxidase activity in haploid callus cultured on shoot inducing medium are

Table : 59 : Growth, Polyphenols and progressive changes in the Activity of Peroxidase, IAA Oxidase, MDH and PAL during shoot differentiation from Haploid callus tissues of *N. tabacum* L. var. Anand-2.

Medium : MS + 0.3 mg/l IAA + 3% sucrose

Incubation : 9 days at 26±2° in continuous light

Inoculum : 300±30 mg fresh tissue

Day	Fresh weight mg/cult.	Dry weight mg/cult.	PEROXIDASE		IAA OXIDASE		MDH		PAL		PHENOLICS	
			units/ cult.	units/mg protein	units/ cult.	units/mg protein	units/ cult.	units/mg protein	units/ cult.	units/mg protein	mg/ cult.	mg%
0	300 (±30)	12 (±3)	0.98	0.86	0.48	0.42	1.05	0.92	0.28	0.25	0.13	1.12
3	492.4 (±36.0)	21.5 (±3.1)	1.87	0.91	5.49	2.67	6.15	3.00	0.21	0.10	0.11	0.52
6	545.1 (±28.2)	39.4 (±6.0)	2.22	1.08	11.61	5.64	16.48	8.00	1.73	0.84	1.35	3.45
9	832.6 (±42.7)	59.7 (±5.4)	5.07	1.11	12.43	2.72	50.11	11.00	1.10	0.24	0.57	0.96

Figures in parenthesis represent standard error.

Data represented is average of 5 replicates.

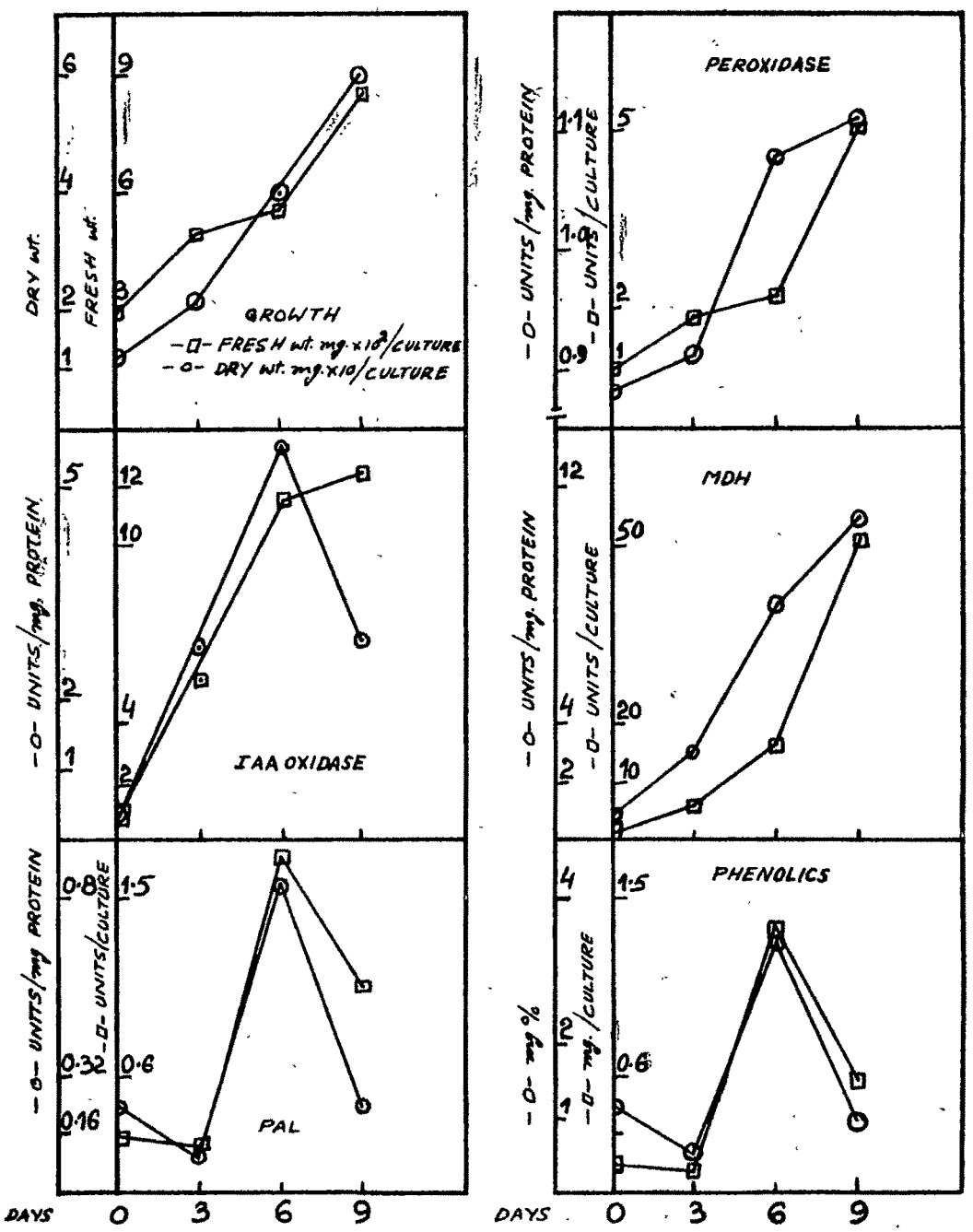


FIG. 121.

Experimental Details as in Table 59.

illustrated in Fig. 121 and Table 59.

The total IAA Oxidase activity in haploid callus increased right from day O. The increase was rapid and linear upto day 6. Thereof, the rate of increase of activity slowed down considerably, before reaching the peak value on day 9. Specific IAA Oxidase activity on the same medium reached its peak activity on day 6 after sharp and linear increase in activity between days O and 6. Thereafter the specific activity declined sharply till day 9 (Fig. 121, Table 59).

The IAA Oxidase activity per unit protein was on rapid decline between days 6 and 9 i.e. the days preceding shoot differentiation.

(d) MDH :

The progressive changes of the total and specific MDH activity in haploid tobacco tissues cultured on shoot differentiating medium, are illustrated in Fig. 121 and Table 59.

The total and specific MDH activity increased from the very outset. The MDH activity on both the accounts increased between days O and 9 to reach peak values (Fig. 121, Table 59).

The MDH activity per unit protein enhanced during the

days immediately preceding shoot differentiation i.e. between days 6 and 9.

(e) PAL :

The progressive changes of total and specific PAL activity in haploid callus cultured on shoot differentiating medium, are illustrated in Fig. 121 and Table 59.

Both total and specific PAL activities in haploid callus declined during the initial 3 days of culture. Thereafter the activity increased to reach the peak value on day 6. Between days 6 and 9 the activity was again on decline (Fig. 121, Table 59).

The PAL activity per unit protein was on decline during the days preceding shoot differentiation i.e. between days 6 and 9.

(f) Phenolics :

Progressive changes of accumulation of phenolics in haploid tobacco callus cultured on shoot inducing medium, are presented in Fig. 121 and Table 59.

The phenolic content of haploid callus cultured declined slightly from 0.13 mg/culture on day 0 to 0.11 mg/culture on day 3. Thereafter the peak value of 1.35 mg/culture was

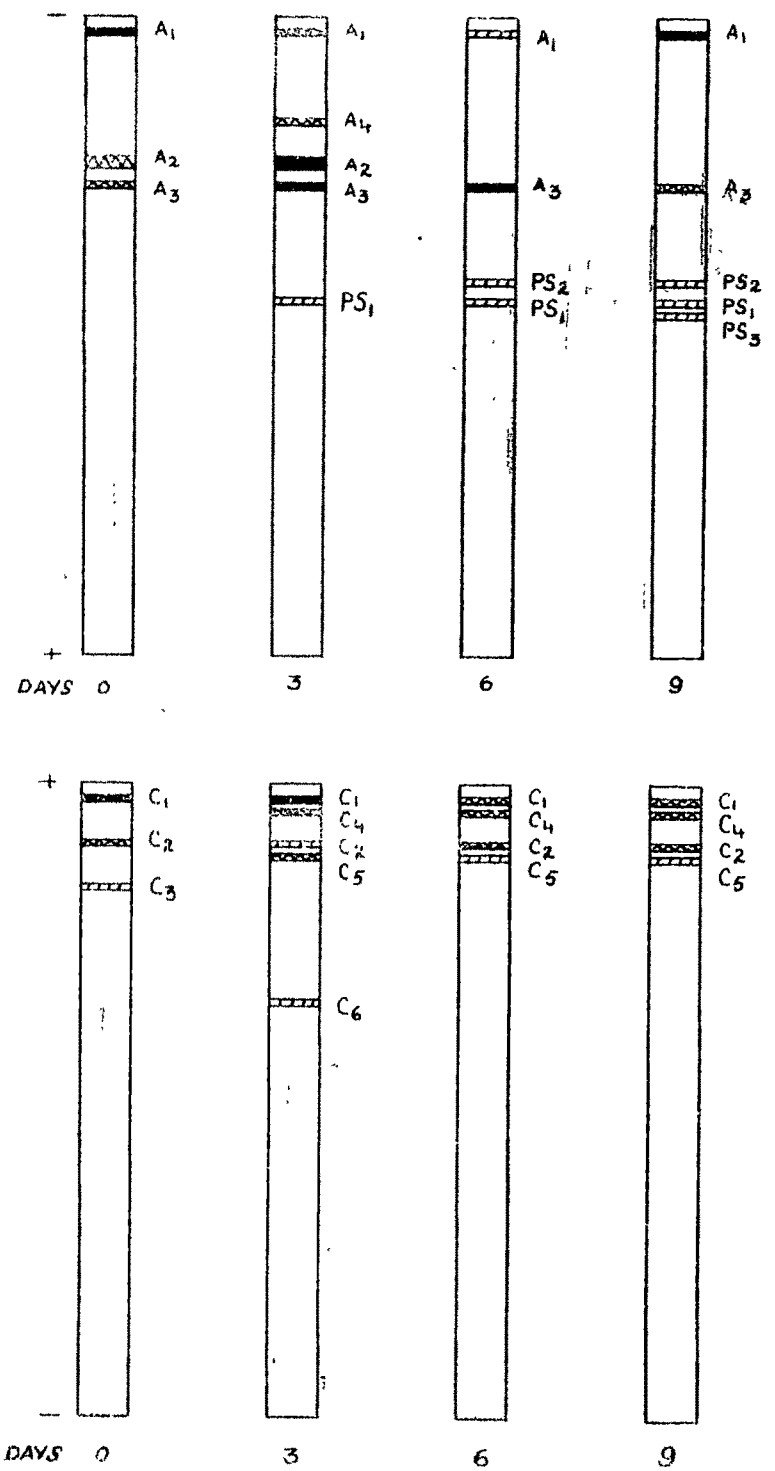
attained on day 6. By day 9 the phenolic content had declined to 0.57 mg/culture. On percentage basis also, the phenolic content declined from 1.12 mg% on day 0 to 0.52 mg% on day 3. The peak value of 3.45 mg% was reached on day 6. Thereafter the phenolic content declined to 0.96 mg% on day 9 (Fig. 121, Table 59).

The phenolic content in haploid tobacco callus declined sharply from 3.45 mg% to 0.96 mg% between days 6 and 9, i.e. the days preceding shoot differentiation.

(g) Peroxidase Isoenzymes :

The progressive changes of anodic and cathodic isoperoxidase banding patterns during shoot differentiation from haploid tobacco callus tissues are illustrated in Fig. 121a.

To begin with the haploid callus demonstrated three anodic isoperoxidases A_1, A_2 and A_3. Of these A_1 and A_3 were synthesized right through the culture period. A_2 appeared only on day 3, and was suppressed for the rest of the culture period. A slow migrating anodic isoperoxidase A_4 was synthesized on day 3. PS_1, a fast migrating anodic isoperoxidase was synthesized on days 3, 6 and 9. Another fast migrating one PS_2 was synthesized on days 6 and 9 of culture. On the penultimate day of culture i.e. day 9, a third fast migrating anodic isoperoxidase PS_3 was synthesized. All the



Anodic and Cathodic Isoperoxidase Patterns.

Experimental Details as in Table 59.

Fig. 121a.

three fast migrating anodic isoperoxidases PS_1, PS_2 and PS_3 were also detected in peroxidase preparations of the regenerated shoots (Fig. 121a).

Of the three original cathodic isoperoxidases C_1 and C_2 persisted right through the culture period. The third one C_3 was suppressed for the entire culture period. Two slow migrating cathodic isoperoxidases C_4 and C_5 were synthesized right through the culture period of 9 days. Another cathodic isoperoxidase C_6 appeared only on day 3 (Fig. 121a).

The first signal for shoot differentiation appeared on day 3 in the shape of anodic isoperoxidase PS_1. Other two anodic isoperoxidases PS_2 and PS_3 were synthesized on days 6 and 9 respectively. All the three isoenzymes were detected in regenerated shoots. No correlation could be detected between cathodic isoperoxidase patterns and shoot differentiation.

Expt. 39. Studies with haploid tobacco callus tissues cultured on shoot inducing medium containing low and high levels of sucrose

Healthy callus masses of haploid tobacco weighing 300±30 mg by fresh weight were cultured on 20 ml of shoot inducing medium (MS basal + 0.3 mg/l IAA) supplemented with low (1%) and high (6%) levels of sucrose.

The culture vessels were incubated at 26±2° in continuous light. Every 3 days, 5 replicates were harvested till day 15 and analysed for growth, enzymes, peroxidase isoenzyme patterns and phenolic accumulation.

(a) Growth :

Growth of haploid callus tissue cultured on the shoot inducing medium supplemented with low (1%) and high(6%) sucrose levels, are illustrated in Figs. 122, 123 and Tables 60, 61.

The growth of haploid callus tissue grown on 1% sucrose containing shoot inducing medium (MS + 0.3 mg/l IAA) did not exhibit any lag phase. The fresh and dry weight increased between days 0 and 15. The fresh weight increased 2.96 fold and dry weight 5.69 fold (Fig. 122, Table 60). On the same medium supplemented with 6% sucrose, growth was more prolific.

Fresh weight increased 5.19 fold and dry weight by 16.11 fold (Fig. 123, Table 61).

The use of low (1%) level of sucrose in shoot inducing medium resulted in suppression of shoot differentiation from haploid callus tissues. On the other hand, incorporation of high (6%) level of sucrose in the shoot inducing medium created a situation conducive to root differentiation rather than shoot differentiation.

(b) Peroxidase :

The progressive changes of peroxidase activity in haploid callus tissue cultured on shoot inducing medium supplemented with low (1%) and high (6%) sucrose levels, are presented in Figs. 122, 123 and Tables 60, 61.

With incorporation of low (1%) sucrose level in the shoot inducing medium, the total peroxidase activity increased linearly till day 6 to reach its peak value. Thereof, it declined continuously till day 15. On the other hand, the specific peroxidase activity reached its peak value on day 3 as a consequence of sharp increase in the activity. The specific activity of peroxidase declined sharply between days 3 and 6, and continued to decline till day 15 (Fig. 122, Table 60).

Table: 60. Growth, Polyphenols and progressive changes in the Activity of Peroxidase, IAA Oxidase, MDH and PAL in haploid callus tissues of N. tabacum L. var. Anand-2 cultured on shoot differentiating medium supplemented with low sucrose level.

Medium : MS + 0.3 mg/l IAA + 1% sucrose

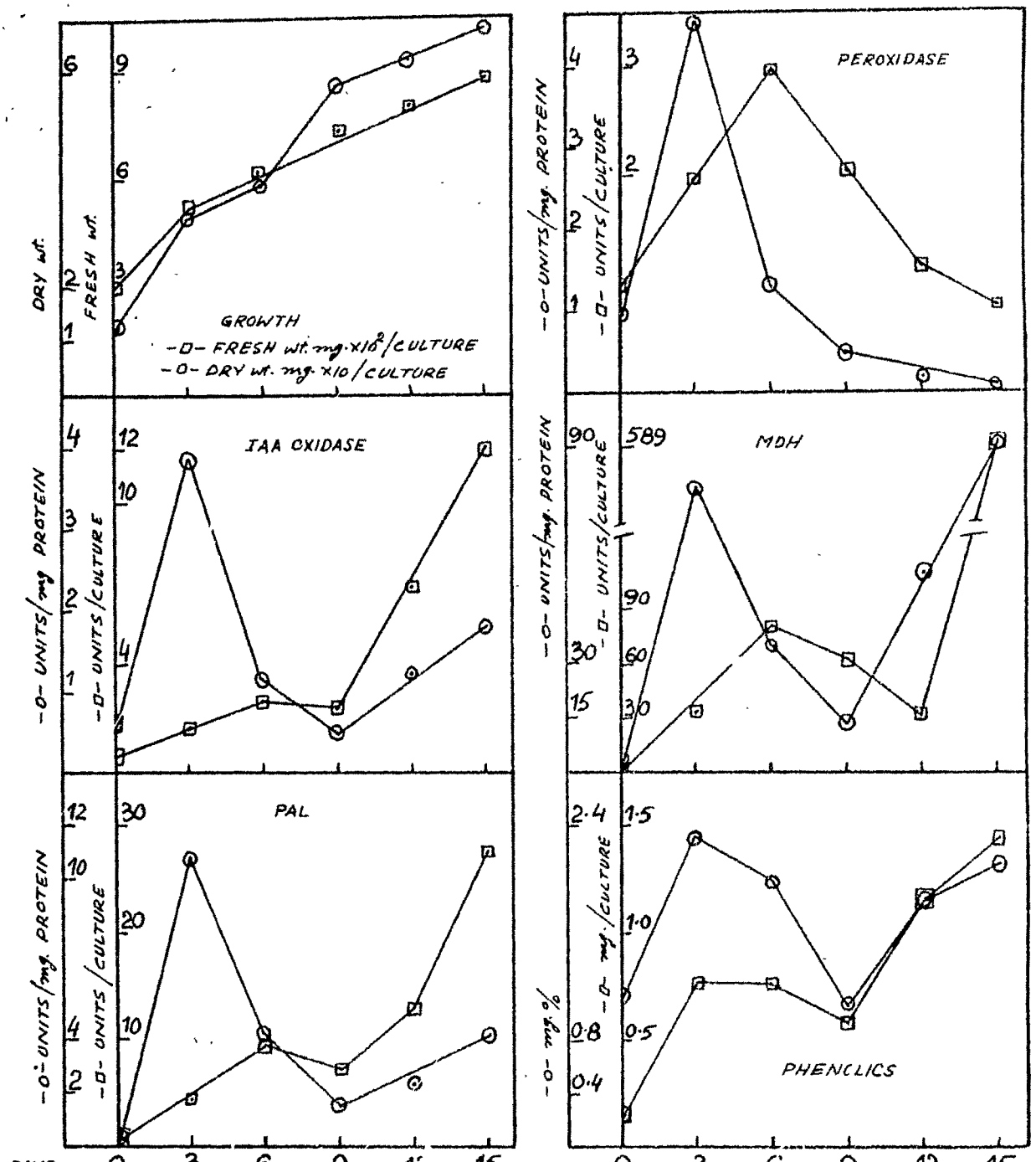
Incubation : 15 days at 26±2° in continuous light

Inoculum : 300±30 mg fresh tissue

Day	Fresh weight mg/cult.	Dry weight mg/cult.	PEROXIDASE		IAA OXIDASE		MDH		PAL		PHENOLICS	
			units/ cult.	units/mg protein	units/ cult.	units/mg protein	units/ cult.	units/mg protein	units/ cult.	units/mg protein	mg/ cult.	mg%
0	300 (±30)	12 (±3)	0.98	0.86	0.48	0.42	1.05	0.92	0.28	0.25	0.13	1.12
3	528.1 (±36.1)	33.5 (±3.1)	2.00	4.64	1.66	3.84	34.56	80.00	4.68	10.85	0.77	2.32
6	615.0 (±32.4)	39.3 (±2.7)	2.94	1.28	2.56	1.11	81.20	35.43	9.56	4.12	0.77	1.97
9	742.6 (±48.7)	57.0 (±3.8)	2.07	0.46	2.33	0.52	62.37	14.00	7.53	1.69	0.58	1.02
12	819.2 (±41.9)	62.8 (±1.7)	1.15	0.21	6.89	1.27	29.96	55.53	13.18	2.44	1.15	1.83
15	889.8 (±86.0)	68.2 (±2.8)	0.80	0.12	12.02	1.83	589.56	89.99	27.68	4.22	1.44	2.11

Figures in parenthesis represent standard error.

Data represented is average of 5 replicates.



Experimental Details as in Table 60.

FIG. 122.

Table: 61. Growth, Polyphenols and progressive changes in the Activity of Peroxidase, IAA Oxidase, MDH and PAL in haploid callus tissues of N. tabacum L. var. Anand-2 cultured on shoot differentiating medium supplemented with high sucrose level.

Medium : MS + 0.3 mg/l IAA + 6% sucrose

Incubation : 15 days at 26± 2° in continuous light

Inoculum : 300±30 mg fresh tissue

Day	Fresh weight mg/cult.	Dry weight mg/cult.	PEROXIDASE		IAA OXIDASE		MDH		PAL		PHENOLICS	
			units/ cult.	units/mg protein	units/ cult.	units/mg protein	units/ cult.	units/mg protein	units/ cult.	units/mg protein	mg/ cult.	mg%
0	300 (±30)	12 (±3)	0.98	0.86	0.48	0.42	1.05	0.92	0.28	0.25	0.13	1.12
3	447.6 (±36.3)	33.4 (±2.2)	0.80	4.95	1.40	8.65	7.15	43.95	7.95	48.89	1.15	3.46
6	480.6 (±40.0)	35.7 (±3.8)	2.73	1.36	2.90	1.44	57.06	28.39	9.61	4.78	0.88	2.48
9	588.6 (±42.1)	73.1 (±6.2)	3.70	2.16	1.24	0.72	10.27	6.00	2.61	1.52	0.79	1.09
12	1086.3 (±47.7)	134.9 (±10.1)	11.31	0.99	11.77	1.03	41.35	3.63	23.22	2.03	2.26	1.68
15	1557.3 (±73.2)	193.4 (±17.7)	3.58	0.22	22.15	1.37	16.36	1.01	41.51	2.57	3.55	1.84

Figures in parenthesis represent standard error.

Data represented is average of 5 replicates.

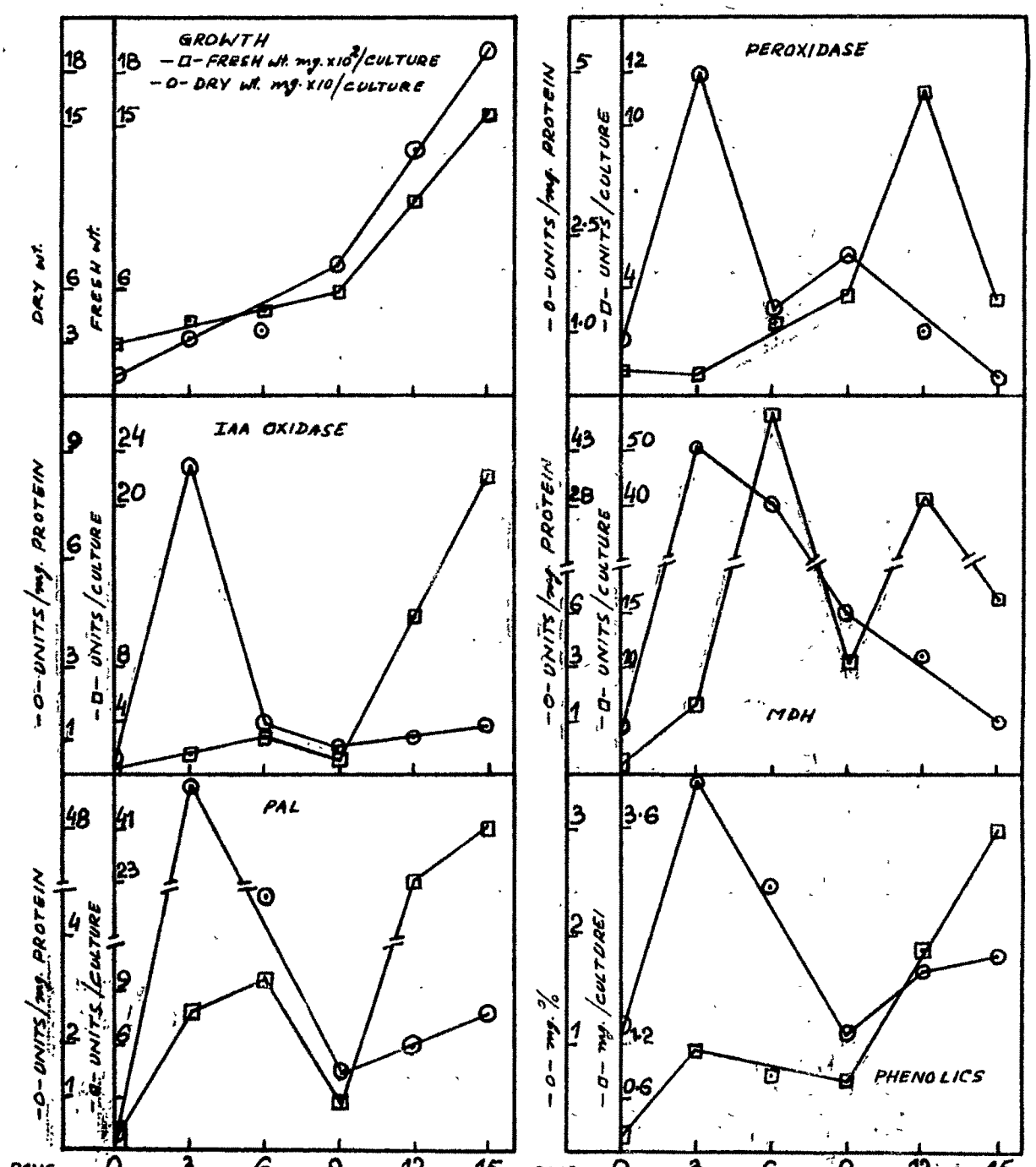


FIG. 123

Experimental Details as in Table 61.

On 6% sucrose containing medium the total peroxidase activity declined slightly between days 0 and 3. Thereafter the activity was on increase till day 12, when the peak value was reached. Between days 12 and 15 the total peroxidase activity dropped sharply, On the other hand, the specific activity exhibited double peaked pattern of development. Between days 0 and 3 the activity increased sharply to its first peak value. However, by day 6 the specific activity of peroxidase decreased very rapidly. Between days 6 and 9 it increased again reaching the second peak on day 9. Between days 9 and 15 the activity was on linear decline (Fig. 123, Table 61).

The peroxidase activity per unit protein was on increase in haploid callus cultured on standard shoot inducing medium, during days 6-9 i.e. the days immediately preceding shoot differentiation. In contrast on 1% sucrose containing medium during the corresponding period the peroxidase activity per unit protein was on decline. The decline of activity continued beyond day 9. During the corresponding period i.e. between days 6 and 9, the peroxidase activity per unit protein was on increase in haploid callus cultured on 6% sucrose containing medium. However, the enzyme activity was considerably higher during this period as compared with activity on the standard shoot inducing medium. On 6% sucrose containing medium the activity decayed rapidly between days 9

and 15. On this high sucrose containing medium root differentiation occurred on day 12 of culture.

(c) IAA Oxidase :

The progressive changes of total and specific IAA Oxidase activity in haploid callus tissue cultured on shoot inducing medium supplemented with low (1%) and high (6%) levels of sucrose, are illustrated in Figs. 122, 123 and Tables 60, 61.

The total IAA Oxidase activity in haploid callus cultured on 1% sucrose containing medium increased slowly and linearly till day 6. Between days 6 and 9 the activity, however, declined slightly. This decay in the activity was followed by a sharp and linear increase in the enzyme activity till day 15. The specific activity, on the other hand, increased sharply between days 0 and 3, registering its peak value. Between days 3 and 9 the enzyme activity decayed rapidly. However, it increased thereof linearly till day 15 (Fig. 122, Table 60). Though on standard shoot inducing medium also, the enzyme activity per unit protein decayed between days 6 and 9. It nevertheless, was at least 5 times higher on standard shoot inducing medium than on 1% sucrose medium during the corresponding period. Days 6-9 were characterized by shoot differentiation from haploid callus

on standard shoot inducing medium.

The total and specific activities of IAA Oxidase in haploid callus cultured on 6% sucrose containing medium, essentially followed similar developmental pattern as on 1% sucrose medium. The total IAA Oxidase activity increased slowly till day 6, reaching a minor peak value. Thereof till day 9 the activity declined slightly. Thereafter, it picked up again and as a consequence of sharp increase in the activity, registering another peak value on day 15. The specific IAA Oxidase activity, on the other hand, increased sharply between days 0 and 3, reaching its peak value. Thereafter till day 9 the activity was on decline along a steep gradient. Between days 9 and 15 the activity increased again (Fig. 123, Table 61). As on 1% sucrose medium, here also the enzyme activity per unit protein was on decline between days 6 and 9. But the activity was atleast 4 times less on 6% sucrose medium than on standard shoot inducing medium, during the corresponding period. On 6% sucrose medium the enzyme activity was, however, on increase between days 9 and 15. In other words, on this particular medium the enzyme activity started to increase just before root differentiation and continued to increase even after.

(d) MDH :

The progressive changes of total and specific MDH

activity in haploid callus tissue cultured on shoot inducing medium containing low (1%) and high (6%) levels of sucrose, are presented in Figs. 122, 123 and Tables 60,61.

The total MDH activity on 1% sucrose containing medium demonstrated double peaked pattern of development. The first peak value was reached on day 6 and the second on day 15. The specific MDH activity also registered two peak values during the course of 15 days culture. The first peak was attained on day 3 and the second on day 15 (Fig. 122, Table 60). In contrast with MDH activity per unit protein on standard shoot inducing medium, where the activity increased during days immediately preceding shoot differentiation (days 6-9), on 1% sucrose medium the activity during the corresponding period was on decline. Furthermore, during the same period the MDH activity was considerably higher on 1% sucrose medium than on standard shoot inducing medium.

As in the previous instance, the total MDH activity on 6% sucrose medium also attained two peak values during the culture period, one each on days 6 and 12. The specific MDH activity increased between days 0 and 3, to reach its peak value. Thereafter till day 15 the activity was on decline (Fig. 123, Table 61). On 6% sucrose medium the MDH activity per unit protein declined very sharply between days 6 and 9, whereas, during the corresponding period it increased on

standard shoot inducing medium. The activity on 6% sucrose medium continued to decline till day 15 and also roots were differentiated on day 12 of culture.

(e) PAL :

The progressive changes of PAL activity in haploid callus tissue cultured on shoot inducing medium containing low (1%) and high (6%) sucrose levels, are presented in Figs. 122, 123 and Tables 60, 61.

The total PAL activity in haploid tobacco callus cultured on 1% sucrose containing medium attained two peak values during the culture period, one each on days 6 and 15. The specific PAL activity also demonstrated two peaked pattern of development, but the peak values were reached on days 3 and 15 (Fig. 122, Table 60). Though PAL activity per unit protein was on decline between days 6 and 9 as also on standard shoot inducing medium, the activity was, nevertheless, considerably higher on 1% sucrose medium.

On 6% sucrose medium, the total PAL activity attained two peak values during the culture period, one each on days 6 and 15. The specific PAL activity shot up during the initial 3 days of culture. Thereafter till day 9 it was on decline along a sharp gradient. Between days 9 and 15 the activity increased again to reach the second peak value

(Fig. 123, Table 61). As in the previous case, though PAL activity per unit protein was on decline between days 6 and 9 on standard shoot inducing medium as well as on 6% sucrose containing medium, the activity was about 6 times higher on the latter medium. However, the enzyme activity increased between days 9 and 15 on 6% sucrose medium and also roots were differentiated on day 12 of culture.

(f) Phenolics :

The progressive changes of phenolic accumulation in haploid callus cultured on shoot inducing medium supplemented with low (1%) and high (6%) levels of sucrose, are illustrated in Figs. 122, 123 and Tables 60, 61.

On culture basis the phenolic content in haploid callus cultured on 1% sucrose containing medium increased from 0.13 mg/culture on day 0 to 0.77 mg/culture on day 3. Thereof, the phenolic content remained stable till day 6, and then declined to 0.58 mg/culture by day 9. Between days 9 and 15 it, however, increased again to reach 1.44 mg/culture on day 15. On percentage basis the phenolic content increased from 1.12 mg% on day 0 to 2.32 mg% on day 3. Between days 3 and 9 the phenolic content declined to 1.02 mg%. Thereof it increased to 2.11 mg% by day 15 (Fig. 122, Table 60). The phenolic content on percentage basis declined between days 6

and 9 on standard shoot inducing medium as well as on the same medium but containing only 1% sucrose. On the former the phenolic content during this three day period declined from 3.45 mg% to 0.96 mg%, whereas on the latter it declined from 1.97 mg% to 1.02 mg%. Hence, during this period of 3 days (i.e. days 6 to 9) which was characterized by the differentiation of shoots from haploid tobacco callus on shoot inducing medium, the phenolic content declined with much more rapidly on standard shoot inducing medium than on 1% sucrose medium.

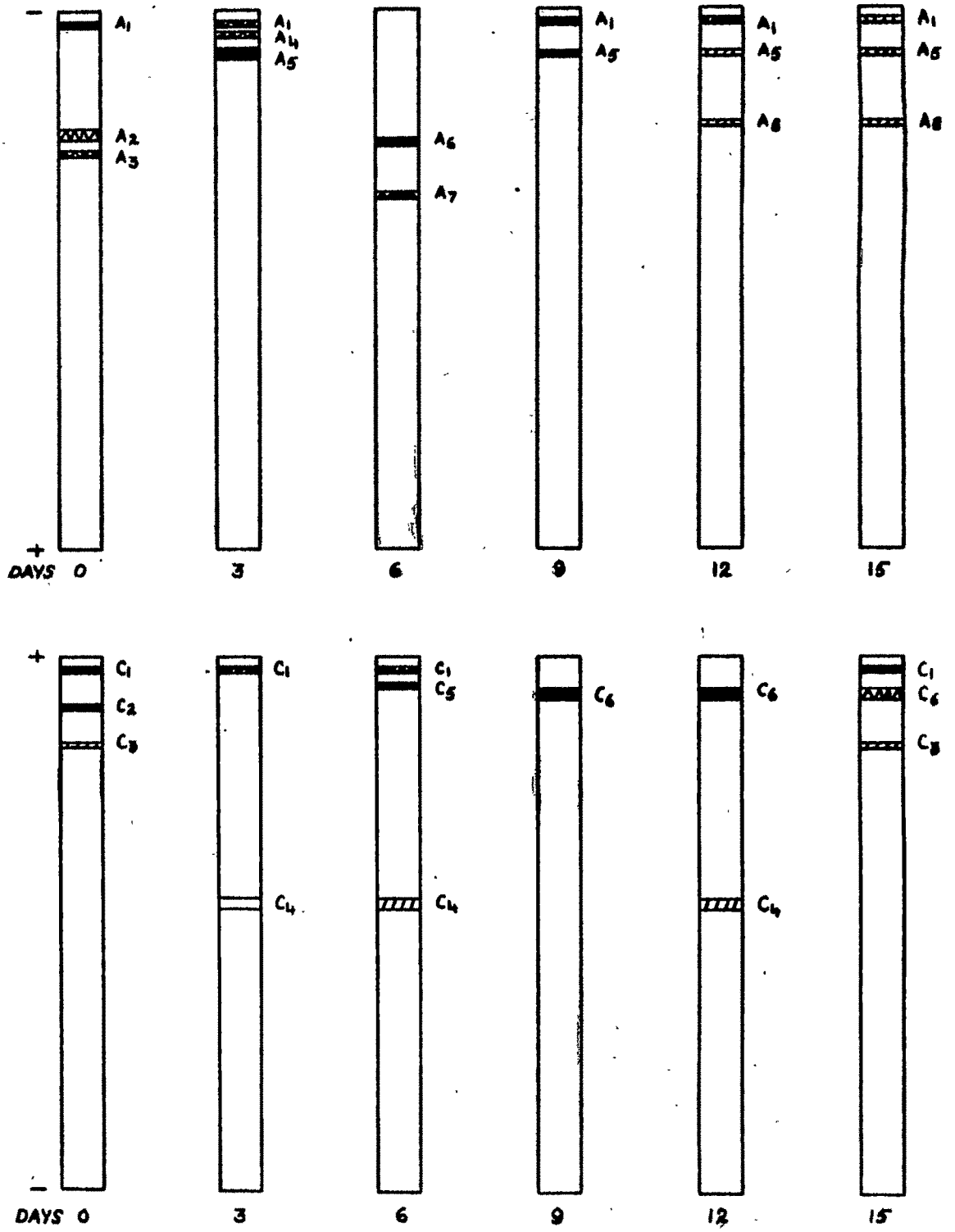
On 6% sucrose medium the phenolic content increased from 0.13 mg/culture on day 0 to 1.15 mg/culture on day 3. Between days 3 and 9 it declined to 0.79 mg/culture. The phenolics accumulated rapidly between day 9 and 15, reaching the value of 3.35 mg/culture on day 15. On percentage basis, peak phenolic accumulation was reached on day 3. Between days 0 and 3 it increased from 1.12 mg% to 3.46 mg%. Thereof, the phenolic content decreased to 1.09 mg% by day 9. Between days 9 and 15 it, however, increased again, reaching the value of 1.84 mg% on day 15 (Fig. 123, Table 61). As in the previous case, here too on 6% sucrose medium the phenolic content on percentage basis declined from 2.48 mg% on day 6 to 1.09 mg% on day 9. On standard shoot inducing medium also the phenolic content declined from 3.45 mg% to 0.96 mg%

during the corresponding period and was followed by shoot differentiation from haploid tobacco callus tissue. On 6% sucrose medium the phenolic content increased between days 9 and 15 from 1.09 mg% to 1.84 mg% and was accompanied by differentiation of roots.

(g) Peroxidase Isoenzymes :

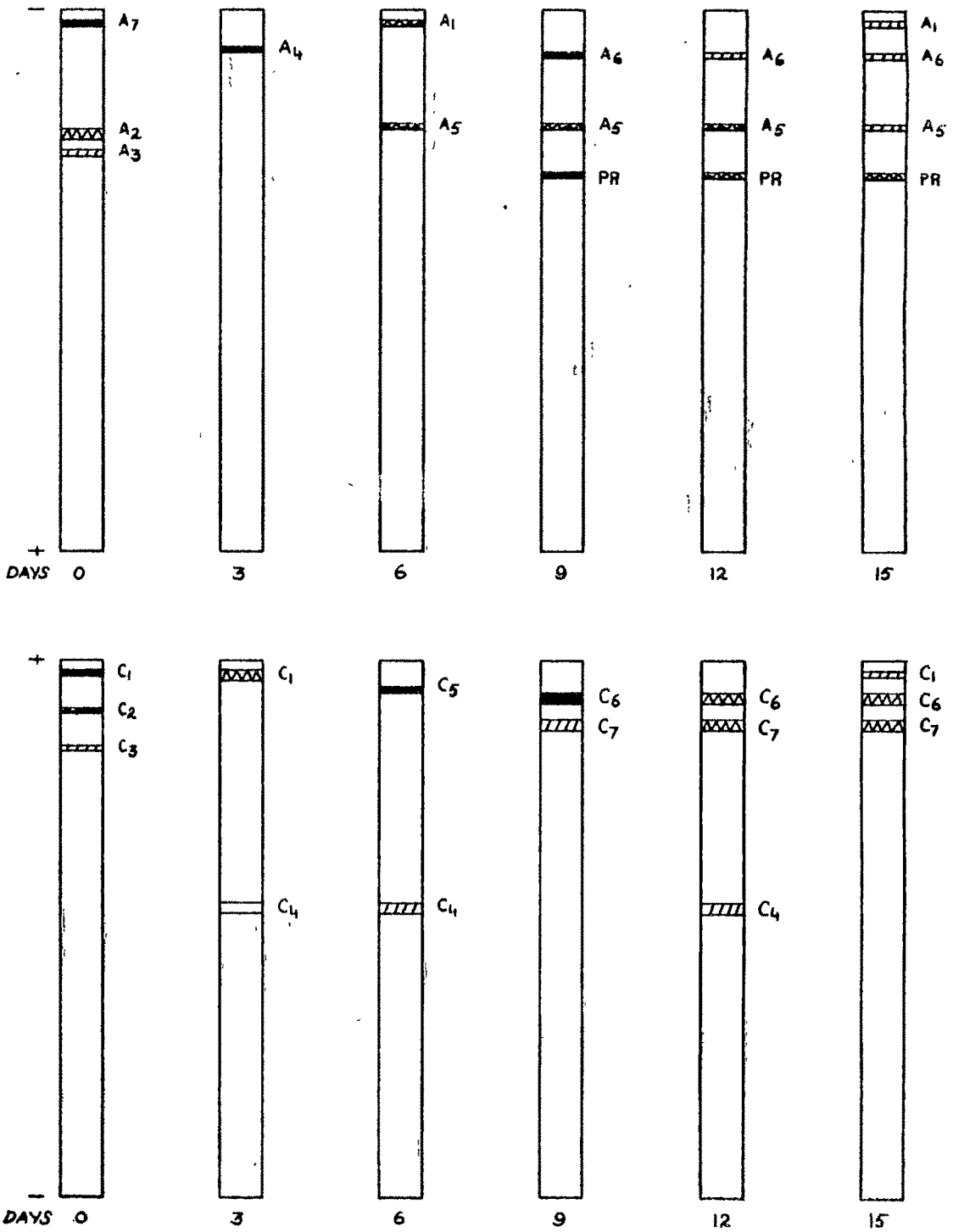
The progressive changes of anodic and cathodic isoperoxidase banding patterns in haploid callus tissue cultured on shoot inducing medium supplemented with low (1%) and high (6%) sucrose levels, are illustrated in Figs. 122a and 123a.

On 1% sucrose containing medium, of the three initial anodic isoperoxidases, only A_1 was synthesized on days 3, 9, 12 and 15. The other two original ones A_2 and A_3 remained suppressed throughout the culture period. During the 15 day culture period 5 slow migrating anodic isoperoxidases A_4 - A_8 were synthesized. A_4 was synthesized on day 3, A_5 on days 9, 12 and 15, A_6 and A_7 on day 6, and A_8 on days 12 and 15. Of the three initial cathodic isoperoxidases, C_2 remained suppressed right through the culture period. C_1, however, appeared on days 3, 6 and 15, and C_3 on day 15. One fast migrating cathodic isoperoxidase C_4 was synthesized on days 3, 6 and 12. Two slow migrating cathodic isoperoxidases C_5 and C_6 were also synthesized. C_5 was synthesized on day 6 and C_6 on days 9, 12 and 15 (Fig. 122a). Of significance



Anodic and Cathodic Isoperoxidase Patterns. Experimental Details as in Table 60.

FIG. 122a



Anodic and Cathodic Isoperoxidase Patterns.
Experimental Details as in Table 61.

FIG. 123a.

was the absence of anodic isoperoxidases PS_1, PS_2 and PS_3, which preceded differentiation of shoots on standard shoot inducing medium.

On 6% sucrose containing medium, of the three initial anodic isoperoxidases, A_2 and A_3 remained suppressed during the entire duration of culture period. A_1 was, however, synthesized on days 6 and 15. Three slow migrating anodic isoperoxidases A_4 - A_6 were synthesized during the culture period. A_4 was synthesized on day 3, A_5 on days 6, 9, 12 and 15, and A_6 on days 9, 12 and 15. Prior to the differentiation of roots on day 12, the root isoperoxidase 'PR' was synthesized on day 9 and continued to appear on days 12 and 15. The anodic isoperoxidase 'PR' was also detected in peroxidase preparation of regenerated roots. The initial cathodic isoperoxidases C_2 and C_3 were not synthesized at any time during the culture period. The third one C_1 was, however, synthesized on days 3 and 15. A fast migrating isoperoxidase C_4 was synthesized on days 3, 6 and 15. Three slow migrating ones C_5 - C_7 were also synthesized. C_5 was synthesized on day 6, and C_6 and C_7 on days 9, 12 and 15 (Fig. 123a). No correspondence was found between root differentiation and cathodic isoperoxidase pattern.

It is noteworthy that with the use of low (1%) sucrose level in the shoot inducing medium, in place of standard

3% sucrose used in the standard shoot inducing medium, the differentiation of shoots was completely arrested as was the synthesis of shoot isoperoxidases PS_1, PS_2 and PS_3. On the other hand, when the sucrose level was increased to 6% in the shoot inducing medium, it no longer favoured shoot differentiation. However, the conditions became favourable for the differentiation of roots, which was preceded by the synthesis of root isoperoxidase "PR". On this medium the synthesis of shoot peroxidases PS_1, PS_2 and PS_3 was also repressed.

Expt. 40. Studies with haploid callus cultured on shoot inducing medium supplemented with various phenolic acids

Healthy callus masses of haploid tobacco weighing 300±30 mg by fresh weight were cultured on 20 ml of the shoot inducing medium (MS basal + 0.3 mg/l IAA + 3% sucrose) and supplemented with various phenolic acids individually. The phenolic acids and their concentrations tested were :

(a) t-cinnamic acid	100 µM
(b) t-cinnamic acid	500 µM
(c) caffeic acid	100 µM
(d) caffeic acid	500 µM
(e) ferulic acid	100 µM
(f) ferulic acid	500 µM
(g) p-hydroxybenzoic acid	1.0 µM
(h) p-hydroxybenzoic acid	10.0 µM

The culture vessels were incubated at 26±2° in continuous light for a period of 9 days. Every 3 days, 5 replicates were harvested and analysed for growth, enzymes, isoperoxidase banding patterns and phenolic content.

(a) Growth :

Growth of the haploid callus cultured on shoot inducing

medium supplemented with various phenolic acids and expressed as increase in fresh and dry weight are expressed in Figs. 124-131 and Tables 62-69.

Of the phenolic acids tested, caffeic acid (100 μM), ferulic acid (100, 500 μM) and p-hydroxybenzoic acid (1.0 μM were stimulatory to growth. Maximum growth increase of 4.21 and 8.74 fold on fresh and dry weight respectively was recorded on 1.0 μM p-hydroxybenzoic acid containing medium.

Incorporation into the medium, of t-cinnamic acid at 100 (a) and 500 μM (b), resulted in suppression of growth as compared with that on standard shoot inducing medium. The growth inhibition was much more pronounced with the use of 100 μM t-cinnamic acid in the medium. Respective fresh and dry weight increases on this medium during the 9 day culture period were 2.13 and 3.66 fold (Fig. 124, Table 62). The fold-wise fresh and dry weight increase on 500 μM t-cinnamic acid medium was 2.31 and 4.30 respectively (Fig. 125, Table 63).

Incorporation of 100 μM t-cinnamic acid into the shoot inducing medium brought about complete inhibition of shoot differentiation from haploid callus. However, the increase of t-cinnamic acid level to 500 μM in the same medium created a situation which was favourable for the differentiation of roots rather than shoots. On this particular medium root

differentiation occurred on day 6 of culture with about 50 per cent frequency.

Addition of 100 μM caffeic acid into the shoot inducing medium (c) stimulated growth as compared with standard shoot inducing medium. The respective fresh and dry weight increase was 3.01 and 5.65 fold (Fig. 126, Table 64). Increase of caffeic acid to 500 μM in the same medium (d) resulted in inhibition of growth in comparison with the standard shoot inducing medium. The fresh weight increased 2.06 fold and dry weight by 3.75 fold (Fig. 127, Table 65).

Addition of 100 μM caffeic acid into the shoot inducing medium proved conducive to root differentiation rather than shoot differentiation. The roots were differentiated on day 9 of culture with about 50 per cent frequency. Higher concentration of caffeic acid (500 μM) in the medium blocked organogenetic expression from haploid callus.

Incorporation of ferulic acid at both the levels tested stimulated growth. On 100 μM ferulic acid medium (e) the respective fresh and dry weight increase was 3.26 and 7.17 fold (Fig. 128, Table 66). On 500 μM ferulic acid medium (f) the fresh weight increased 3.32 fold and dry weight increased 7.75 fold (Fig. 129, Table 67).

Incorporation of ferulic acid (100, 500 μM) into the

shoot inducing medium inhibited shoot differentiation in its entirity.

Incorporation of 1.0 µM p-hydroxybenzoic acid (g) into the shoot inducing medium stimulated growth of haploid tobacco callus. Respective fresh and dry weight increase was 4.21 and 8.74 fold (Fig. 130, Table 68). Increase of p-hydroxybenzoic acid to 10.0 µM (h) in the same medium brought about marginal inhibition of growth in comparison with standard shoot inducing medium. The fresh weight increased 2.39 fold and dry weight by 4.95 fold (Fig. 131, Table 69).

Addition of p-hydroxybenzoic acid at both the levels into the shoot inducing medium, inhibited shoot differentiation from haploid tobacco callus.

(b) Peroxidase :

Progressive changes of total and specific peroxidase activity in haploid callus cultured on shoot inducing medium supplemented with various phenolic acids, are illustrated in Figs. 124-131 and Tables 62-69.

The total peroxidase activity in haploid callus cultured on 100 µM t-cinnamic acid medium (a), increased marginally between days 0 and 3. Thereafter till day 6 the activity

declined sharply, only to increase again and reach its peak value on day 9. The specific peroxidase activity declined considerably during the initial 3 days of culture. Thereof, the activity increased rapidly and linearly till day 9 (Fig. 124, Table 62).

On 500 µM t-cinnamic acid medium (b) the total peroxidase activity exhibited a slight decline during the initial 3 days of culture. The activity continued to decline till day 6, but at a faster rate. Thereafter it, however, increased sharply to reach its peak value on day 9. The specific peroxidase activity, on the other hand, was on decline continuously right through the culture period of 9 days. The rate of decline of enzyme activity was rapid between days 0 and 6. Thereof the activity decayed at a slower rate till day 9 (Fig. 125, Table 63).

The total peroxidase activity in haploid tobacco callus cultured on 100 µM caffeic acid containing medium (c) declined linearly and rapidly during the initial 6 days. Thereafter till day 9 the activity was on increase. The specific peroxidase activity was on decrease through out the culture period. The decline during the initial 3 days was sharp. Thereof it was, however, slow and linear till day 9 (Fig. 126, Table 64).

On 500 µM caffeic acid containing medium (d), the total and specific peroxidase activity exhibited identical developmental pattern. The activity decreased very rapidly during the initial 3 days of culture. By day 6 it had, however, climbed up again, but declined again thereof till day 9 (Fig. 127, Table 65).

With supplementation of 100 µM ferulic acid into the medium (e), the total and specific peroxidase activity exhibited identical developmental pattern. The activity reached two peak values during the culture period. The first peak was attained on day 3 and the second on day 9 (Fig. 128, Table 66).

The total peroxidase activity was on increase from the very outset in haploid tobacco callus cultured on 500 µM ferulic acid containing medium (f). The peak value was reached on day 9. The specific peroxidase activity declined slightly during the initial 3 days of culture. Thereafter it increased rapidly to reach its peak value on day 9 (Fig. 129, Table 67).

The total and specific peroxidase activity demonstrated identical developmental pattern in haploid tobacco callus cultured on 1.0 µM p-hydroxybenzoic acid containing medium (g).

Table: 62. Growth, Polyphenols and progressive changes in the Activity of Peroxidase, IAA Oxidase, MDH and PAL in haploid callus tissues of N. tabacum L. var. Anand-2 cultured on shoot differentiating medium supplemented with 100 uM t-cinnamic acid

Medium : MS + 0.3 mg/l IAA + 100 uM t-cinnamic acid + 3% sucrose

Incubation : 9 days at 26±2° in continuous light

Inoculum : 300±30 mg fresh tissue

Day	Fresh weight mg/cult.	Dry weight mg/cult.	PEROXIDASE		IAA OXIDASE		MDH		PAL		PHENOLICS	
			units/ cult.	units/mg protein	units/ cult.	units/mg protein	units/ cult.	units/mg protein	units/ cult.	units/mg protein	mg/ cult.	mg%
0	300 (±30)	12 (±3)	0.98	0.86	0.48	0.42	1.05	0.92	0.28	0.25	0.13	1.12
3	578.9 (±28.1)	30.2 (±3.0)	1.04	0.48	1.22	0.56	3.85	1.78	5.38	2.49	0.56	1.87
6	601.1 (±42.7)	41.3 (±3.9)	0.48	0.68	1.27	1.79	4.00	5.63	14.69	20.68	1.16	2.83
9	641.0 (±36.6)	44.0 (±4.1)	1.72	0.92	2.66	1.43	13.14	7.04	1.42	0.76	0.71	1.63

Figures in parenthesis represent standard error.

Data represented is average of 5 replicates.

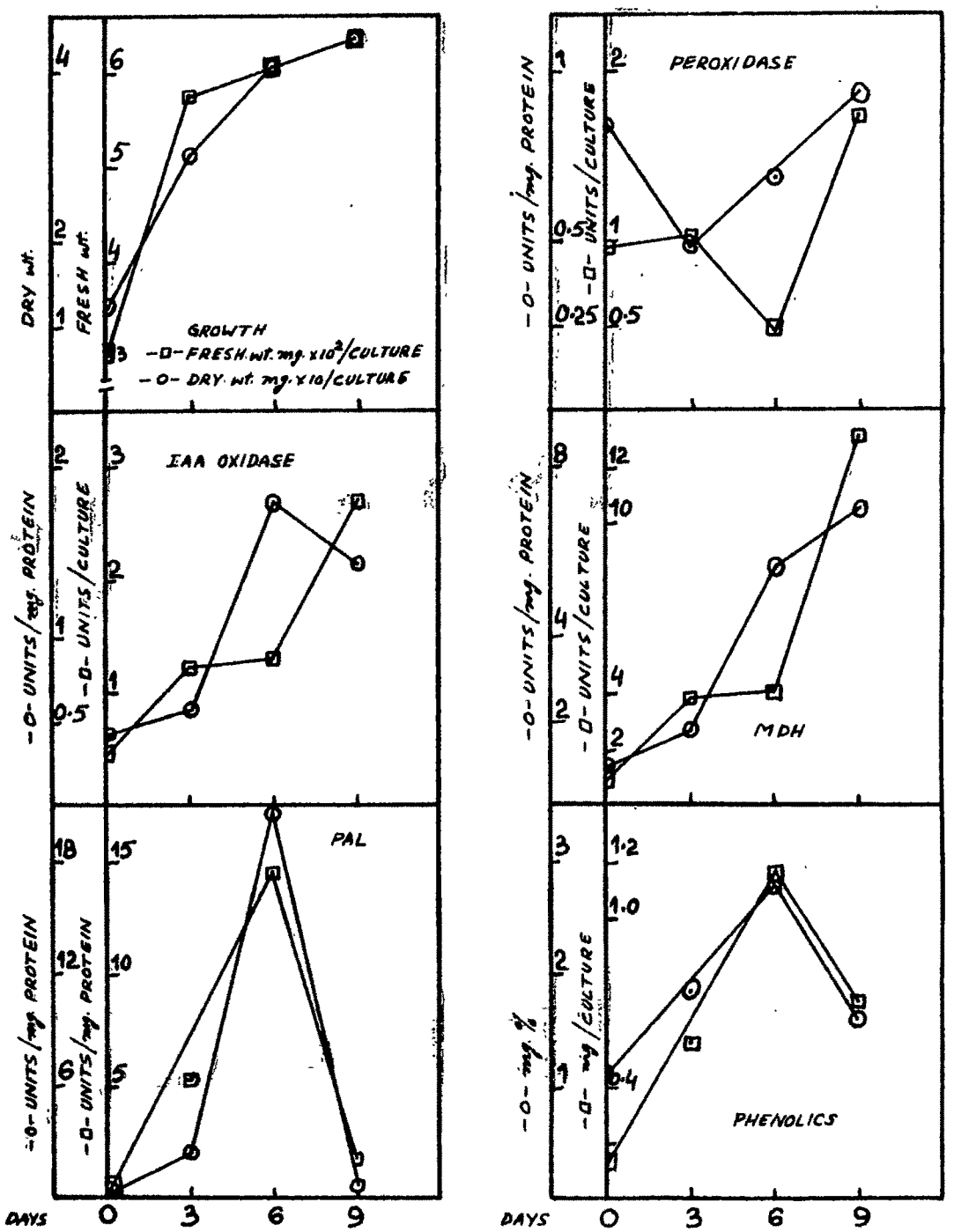


FIG. 124.

Experimental Details as in Table 62.

Table: 63. Growth, Polyphenols and progressive changes in the Activity of Peroxidase, IAA Oxidase, MDH and PAL in haploid callus tissues of N. tabacum L. var. Anand-2 cultured on shoot differentiating medium supplemented with 500 uM t-cinnamic acid.

Medium : MS + 0.3 mg/l IAA + 500 uM t-cinnamic acid + 3% sucrose

Incubation : 9 days at 26±2° in continuous light

Inoculum : 300±30 mg fresh tissue

Day	Fresh weight mg/cult.	Dry weight mg/cult.	PEROXIDASE		IAA OXIDASE		MDH		PAL		PHENOLICS	
			units/ cult.	units/mg protein	units/ cult.	units/mg protein	units/ cult.	units/mg protein	units/ cult.	units/mg protein	mg/ cult.	mg%
0	300 (±30)	12 (±3)	0.98	0.86	0.48	0.42	1.05	0.92	0.28	0.25	0.13	1.12
3	504.1 (±40.2)	29.1 (±2.4)	0.90	0.55	1.58	0.96	1.34	0.81	5.60	3.39	0.79	2.74
6	577.7 (±40.7)	42.9 (±3.6)	0.26	0.38	0.63	0.93	3.84	5.63	7.70	11.28	1.60	3.74
9	695.8 (±38.9)	51.7 (±5.1)	0.93	0.32	4.21	1.44	12.52	4.30	20.09	9.09	1.82	3.53

Figures in parenthesis represent standard error.

Data represented is average of 5 replicates.

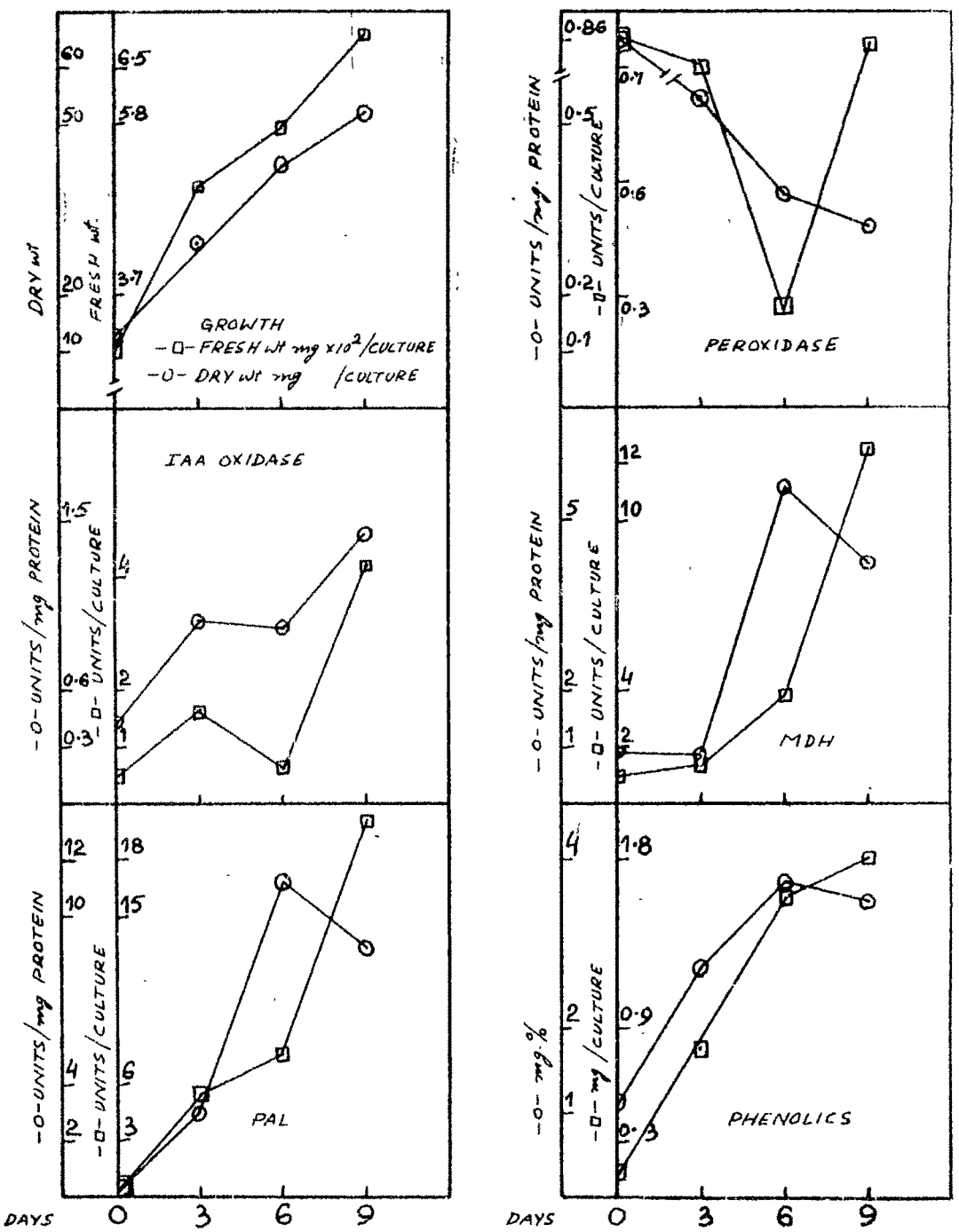


FIG. 125

Experimental Details as in Table 63.

Table: 64. Growth, Polyphenols and progressive changes in the Activity of Peroxidase, IAA Oxidase, MDH and PAL in haploid callus tissues of N. tabacum L. var. Anand-2 cultured on shoot differentiating medium supplemented with 100 uM caffeic acid.

Medium : MS + 0.3 mg/l IAA + 100 uM caffeic acid + 3% sucrose
Incubation : 9 days at 26±2° in continuous light
Inoculum : 300±30 mg fresh tissue

Day	Fresh weight mg/cult.	Dry weight mg/cult.	PEROXIDASE		IAA OXIDASE		MDH		PAL		PHENOLICS	
			units/ cult.	units/mg protein	units/ cult.	units/mg protein	units/ cult.	units/mg protein	units/ cult.	units/mg protein	mg/ cult.	mg%
0	300 (±30)	12 (±3)	0.98	0.86	0.48	0.42	1.05	0.92	0.28	0.25	0.13	1.12
3	713.4 (±38.1)	43.5 (±4.1)	0.64	0.45	0.78	0.55	3.80	2.66	12.67	8.88	0.92	2.12
6	742.7 (±42.3)	55.6 (±5.8)	0.34	0.39	0.81	0.93	7.91	9.02	8.25	9.40	1.92	3.46
9	905.2 (±40.9)	67.9 (±5.2)	0.65	0.36	1.92	1.06	9.95	5.50	22.12	12.22	2.49	3.68

Figures in parenthesis represent standard error.
Data represented is average of 5 replicates.

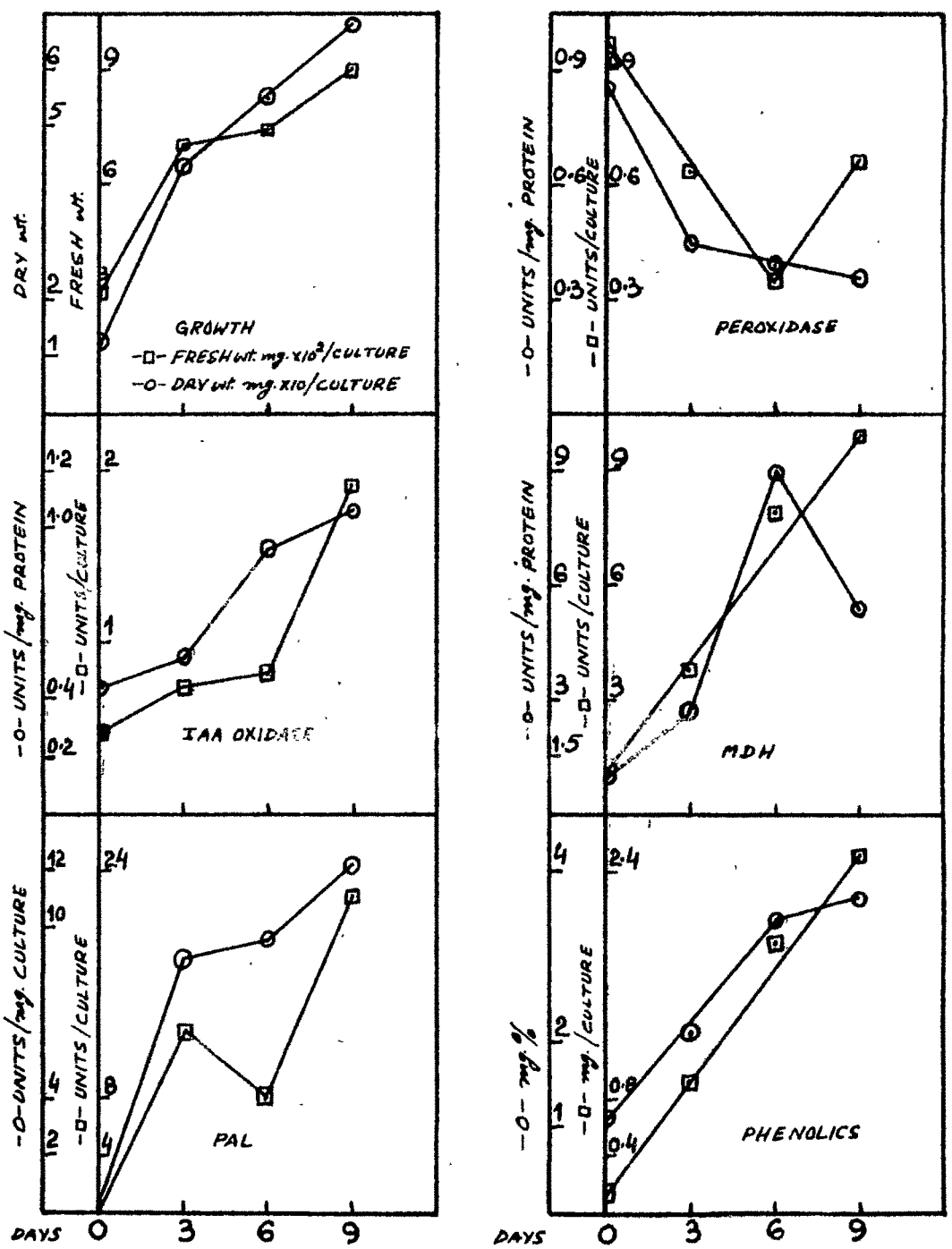


Fig. 126.

Experimental Details as in Table 64.

Table : 65. Growth, Polyphenols and progressive changes in the Activity of Peroxidase, IAA Oxidase, MDH and PAL in haploid callus tissues of N.tabacum L. var. Anand-2 cultured on shoot differentiating medium supplemented with 500 uM caffeic acid.

Medium : MS + 0.3 mg/l IAA + 500 uM caffeic acid + 3% sucrose

Incubation : 9 days at 26±2° in continuous light

Inoculum : 300±30 mg fresh tissue

Day	Fresh weight mg/cult.	Dry weight mg/cult.	PEROXIDASE		IAA OXIDASE		MDH		PAL		PHENOLICS	
			units/ cult.	units/mg protein	units/ cult.	units/mg protein	units/ cult.	units/mg protein	units/ cult.	units/mg protein	mg/ cult.	mg%
0	300 (±30)	12 (±3)	0.98	0.86	0.48	0.42	1.05	0.92	0.28	0.25	0.13	1.12
3	543.0 (±32.0)	35.1 (±3.5)	0.48	0.24	0.59	0.29	2.17	1.07	6.03	2.98	0.69	1.98
6	589.2 (±36.4)	42.9 (±2.9)	0.97	0.82	0.64	0.55	6.28	5.33	6.54	5.55	1.03	2.42
9	618.0 (±36.9)	45.0 (±3.6)	0.83	0.46	5.39	2.99	12.66	7.04	19.22	10.69	1.25	2.78

Figures in parenthesis represent standard error.

Data represented is average of 5 replicates.

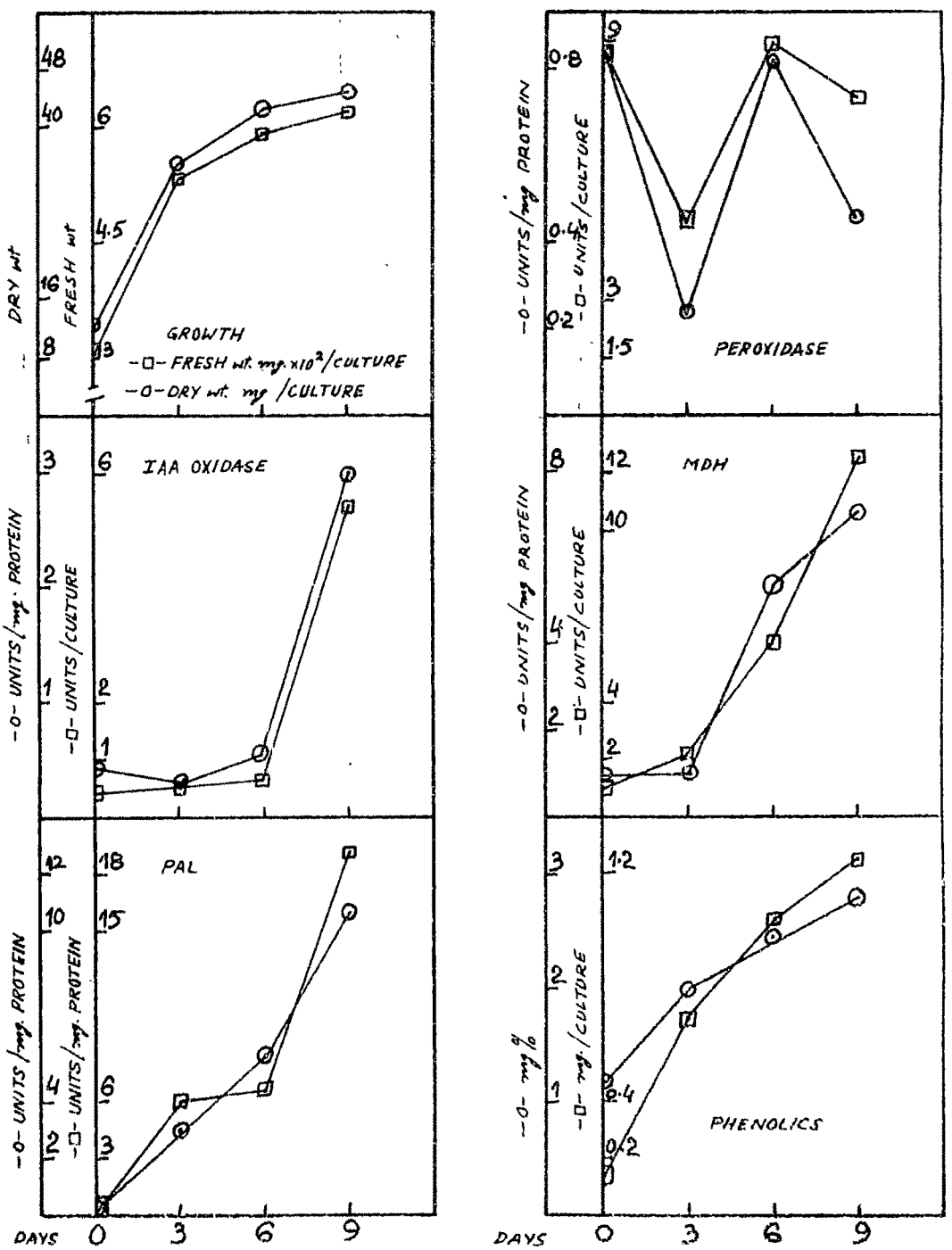


FIG. 127.

Experimental Details as in Table. 65

Table : 66. Growth, Polyphenols and progressive changes in the Activity of Peroxidase, IAA Oxidase, MDH and PAL in haploid callus tissues of N. tabacum L. var. Anand-2 cultured on shoot differentiating medium supplemented with 100 uM Ferulic acid.

Medium : MS + 0.3 mg/l IAA + 100 uM Ferulic acid + 3% sucrose

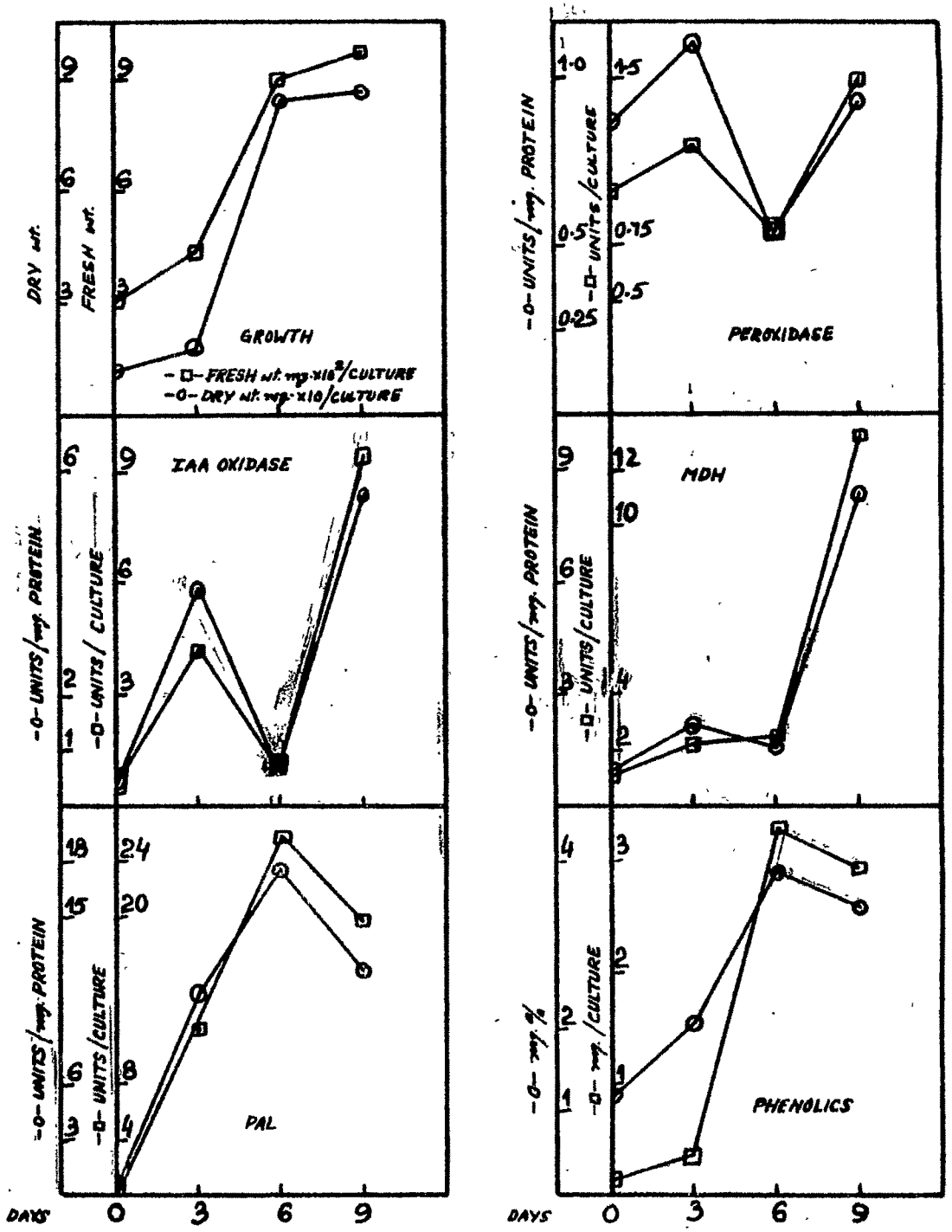
Incubation : 9 days at 26±2° in continuous light

Inoculum : 300±30 mg fresh tissue

Day	Fresh weight mg/cult.	Dry weight mg/cult.	PEROXIDASE		IAA OXIDASE		MDH		PAL		PHENOLICS	
			units/ cult.	units/mg protein	units/ cult.	units/mg protein	units/ cult.	units/mg protein	units/ cult.	units/mg protein	mg/ cult.	mg%
0	300 (±30)	12 (±3)	0.98	0.86	0.48	0.42	1.05	0.92	0.28	0.25	0.13	1.12
3	443.3 (±41.4)	17.4 (±1.2)	1.19	1.10	4.25	3.90	2.36	2.17	11.81	10.86	0.35	2.02
6	906.0 (±56.7)	84.3 (±7.2)	0.78	0.53	0.99	0.67	2.40	1.62	26.16	17.65	3.26	3.87
9	979.3 (±42.3)	86.1 (±8.6)	1.49	0.93	9.38	5.85	13.22	8.25	19.58	12.22	2.93	3.41

Figures in parenthesis represent standard error.

Data represented is average of 5 replicates.



Experimental Details as in Table 66.

FIG. 128

Table : 67. Growth, Polyphenols and progressive changes in the Activity of Peroxidase, IAA Oxidase, MDH and PAL in haploid callus tissues of N. tabacum L. var. Anand-2 cultured on shoot differentiating medium supplemented with 500 uM Ferulic acid.

Medium : MS + 0.3 mg/l IAA + 500 uM Ferulic acid + 3% sucrose

Incubation : 9 days at 26±2° in continuous light

Inoculum : 300±30 mg fresh tissue

Day	Fresh weight mg/cult.	Dry weight mg/cult.	PEROXIDASE		IAA OXIDASE		MDH		PAL		PHENOLICS	
			units/ cult.	units/mg protein	units/ cult.	units/mg protein	units/ cult.	units/mg protein	units/ cult.	units/mg protein	mg/ cult.	mg%
0	300 (±30)	12 (±3)	0.98	0.86	0.48	0.42	1.05	0.92	0.28	0.25	0.13	1.12
3	559.4 (±42.1)	42.8 (±3.2)	1.28	0.79	2.32	1.43	0.74	0.45	14.91	9.16	1.38	3.24
6	583.0 (±40.9)	52.8 (±4.8)	2.42	1.43	1.23	0.72	9.32	5.50	10.35	6.10	1.51	2.86
9	996.6 (±47.3)	90.4 (±7.6)	5.79	2.36	14.88	6.08	15.94	6.51	24.35	9.95	3.32	3.68

Figures in parenthesis represent standard error.

Data represented is average of 5 replicates.

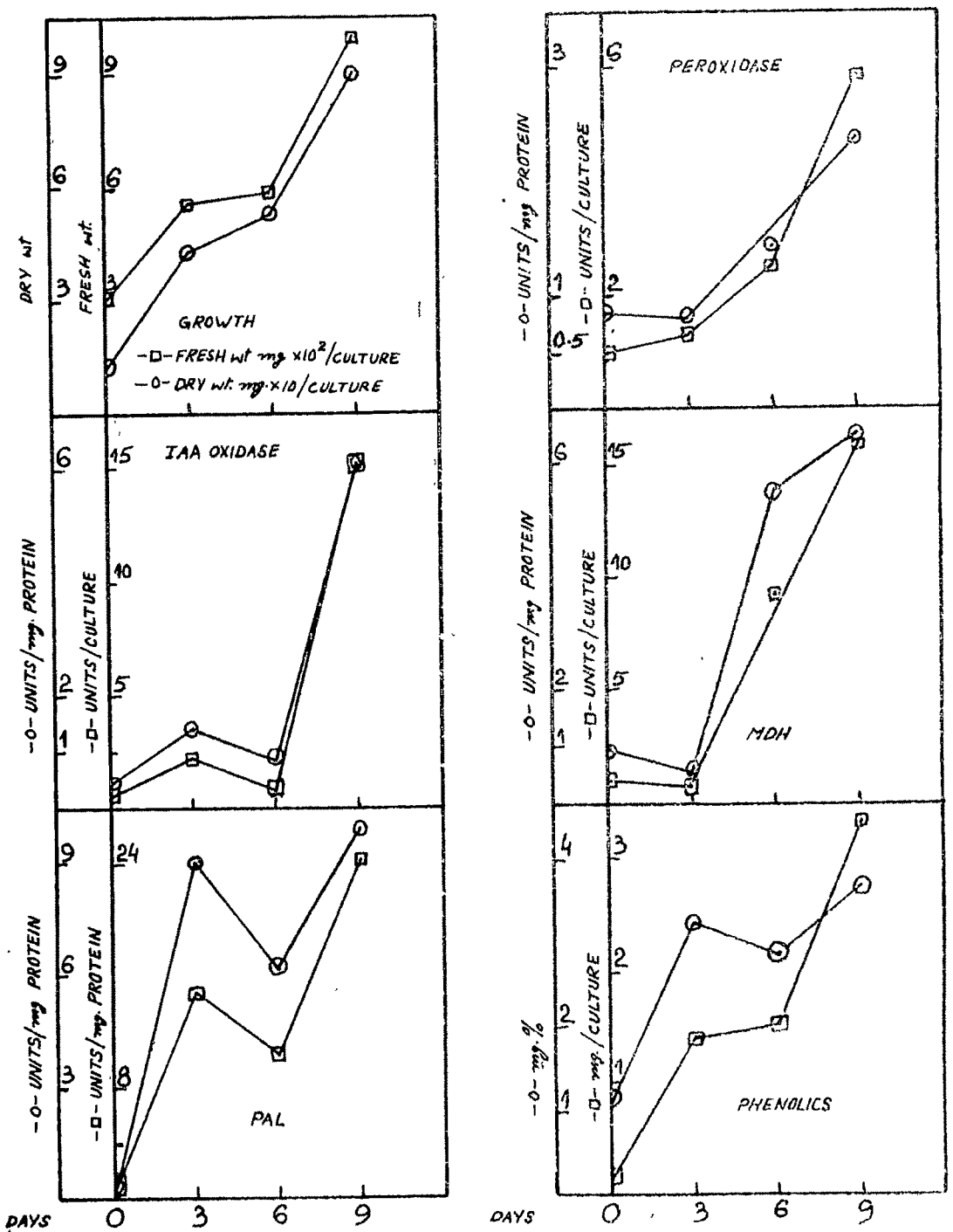


FIG. 12.9.

Experimental Details as in Table 67.

Table : 68. Growth, Polyphenols and progressive changes in the Activity of Peroxidase, IAA Oxidase, MDH and PAL in haploid callus tissues of N. tabacum L. var. Anand-2 cultured on shoot differentiating medium supplemented with 1.0 uM p-hydroxybenzoic acid.

Medium : MS + 0.3 mg/l IAA + 1.0 uM p-hydroxybenzoic acid + 3% sucrose

Incubation : 9 days at 26±2° in continuous light

Inoculum : 300±30 mg fresh tissue

Day	Fresh weight mg/cult.	Dry weight mg/cult.	PEROXIDASE		IAA OXIDASE		MDH		PAL		PHENOLICS	
			units/ cult.	units/mg protein	units/ cult.	units/mg protein	units/ cult.	units/mg protein	units/ cult.	units/mg protein	mg/ cult.	mg%
0	300 (±30)	12 (±3)	0.98	0.86	0.48	0.42	1.05	0.92	0.28	0.25	0.13	1.12
3	479.8 (±36.7)	27.5 (±2.2)	0.67	0.48	3.35	2.40	0.63	0.45	5.86	4.20	0.54	1.98
6	692.6 (±47.2)	57.4 (±4.3)	1.43	0.78	5.17	2.83	6.97	3.82	18.68	10.23	1.78	3.11
9	1264.4 (±80.2)	104.9 (±8.7)	2.83	1.12	8.84	3.94	20.23	8.00	22.46	8.88	2.95	2.82

Figures in parenthesis represent standard error.

Data represented is average of 5 replicates.

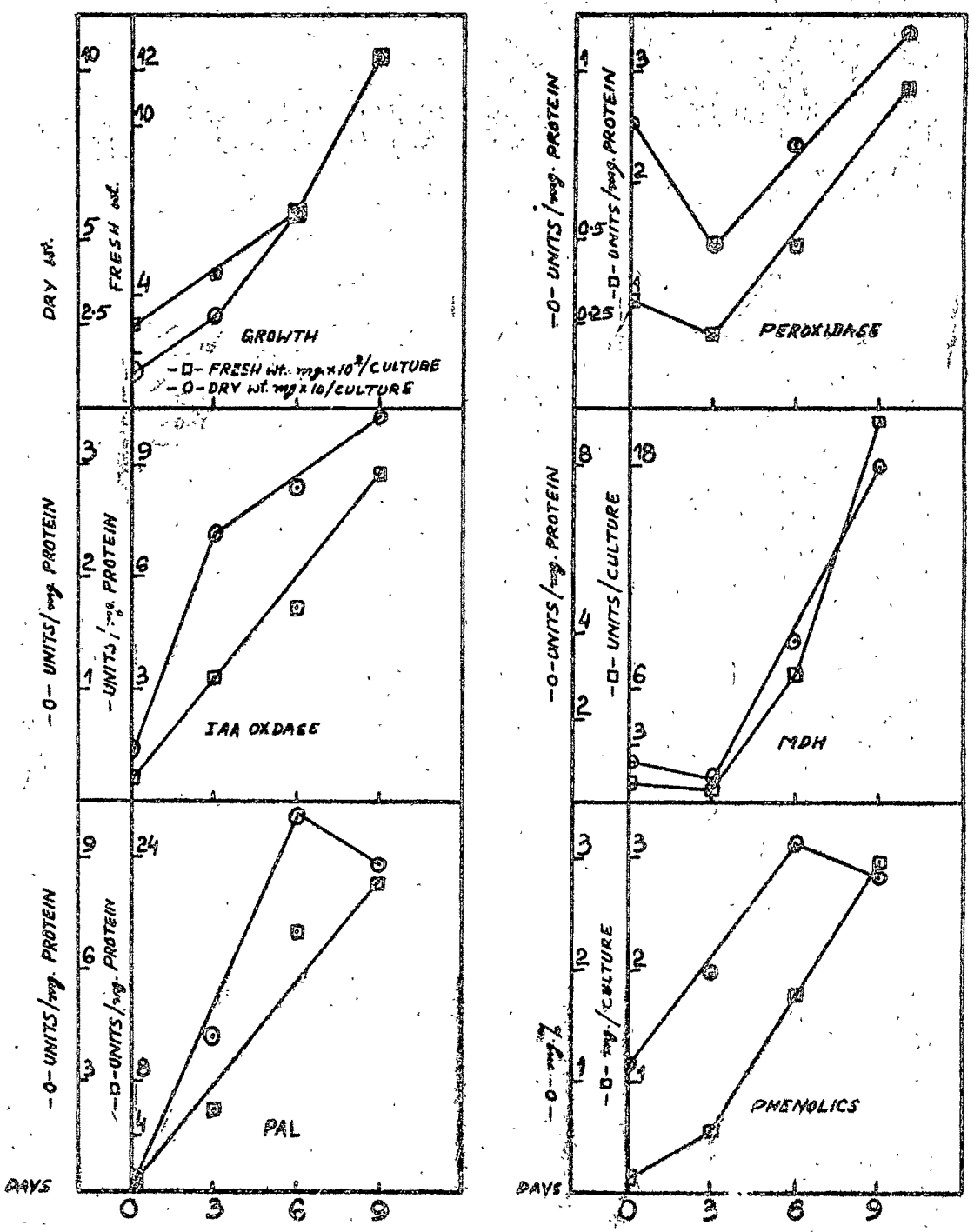


FIG. 130

Experimental Details as in Table 68.

Table : 69. Growth, Polyphenols and progressive changes in the Activity of Peroxidase, IAA Oxidase, MDH and PAL in haploid callus tissues of N. tabacum L. var. Anand-2 cultured on shoot differentiating medium supplemented with 10.0 uM p-hydroxybenzoic acid.

Medium : MS + 0.3 mg/l IAA + 10.0 uM p-hydroxybenzoic acid + 3% sucrose

Incubation : 9 days at 26±2° in continuous light

Inoculum : 300±30 mg fresh tissue

Day	Fresh weight mg/cult.	Dry weight mg/cult.	PEROXIDASE		IAA OXIDASE		MDH		PAL		PHENOLICS	
			units/ cult.	units/mg protein	units/ cult.	units/mg protein	units/ cult.	units/mg protein	units/ cult.	units/mg protein	mg/ cult.	mg%
0	300 (±30)	12 (±3)	0.98	0.86	0.48	0.42	1.05	0.92	0.28	0.25	0.13	1.12
3	513.1 (±41.6)	31.0 (±2.7)	0.66	0.39	1.08	0.64	2.05	1.22	13.67	8.14	0.79	2.58
6	551.9 (±40.2)	45.7 (±4.1)	0.09	0.10	1.73	1.92	0.73	0.81	4.90	5.42	0.90	1.98
9	717.1 (±51.2)	59.5 (±4.3)	4.10	1.36	18.87	6.32	12.90	4.30	15.13	5.04	1.02	1.72

Figures in parenthesis represent standard error.

Data represented is average of 5 replicates.

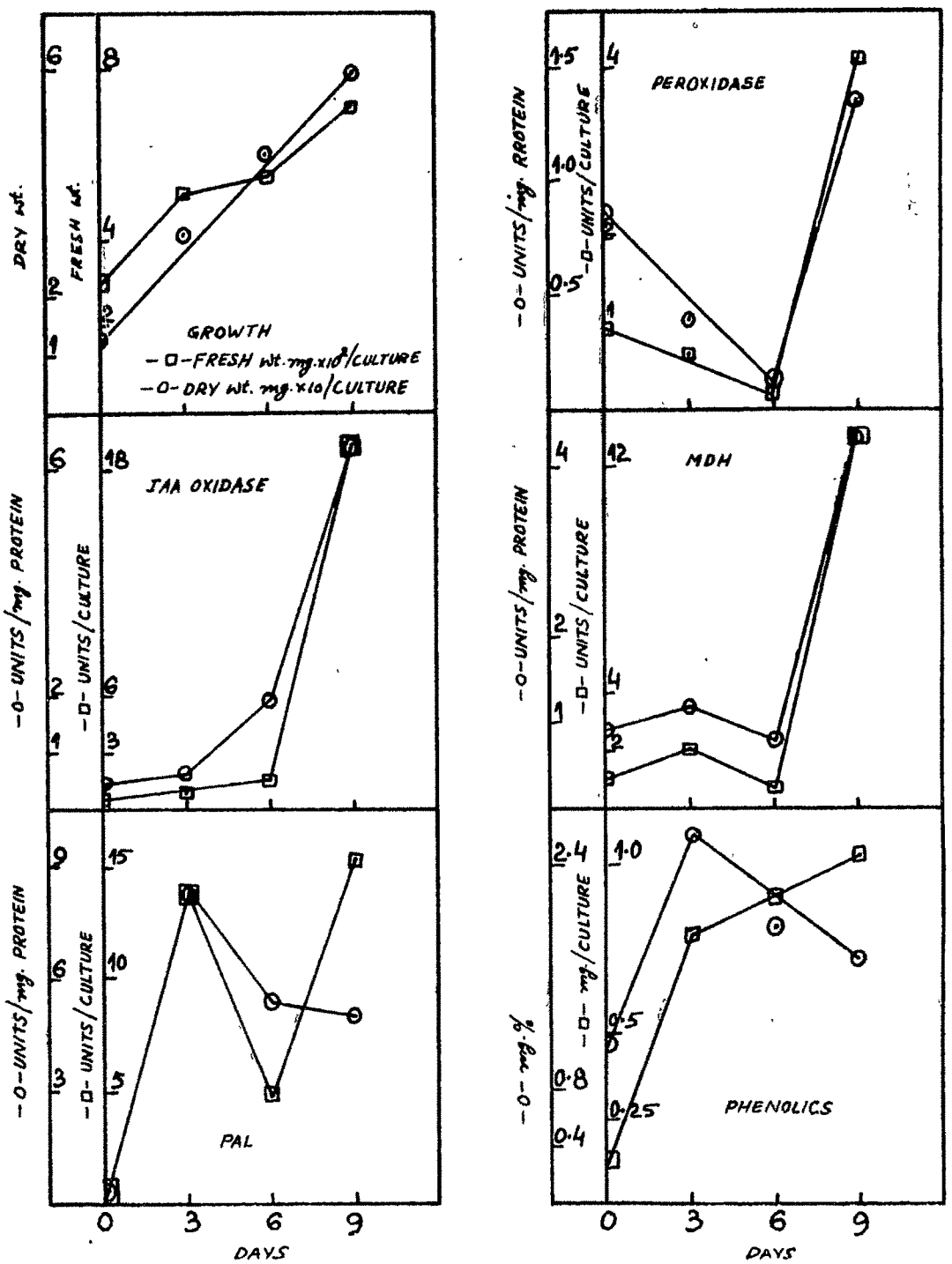


FIG. 131

Experimental Details as in Table 69.

On both the accounts the activity declined between days 0 and 3. Thereafter it increased rapidly and linearly till day 9 (Fig. 130, Table 68).

On 10.0 μM p-hydroxybenzoic acid medium (h) the total and specific activity of peroxidase declined sharply between days 0 and 6. Thereafter the peroxidase activity on both accounts increased sharply till day 9 (Fig. 131, Table 69).

With the exception of t-cinnamic acid (500 μM) and caffeic acid (100, 500 μM), the addition of all other phenolic acids into the shoot inducing medium resulted in increase between days 6 and 9 of peroxidase activity per unit protein. On 500 μM t-cinnamic acid containing medium the decline of activity per unit protein from 0.55 units to 0.38 units between days 3 and 6 was followed by root differentiation on day 6. In a similar fashion on 100 μM caffeic acid medium the enzyme activity declined from 0.45 units on day 3 to 0.36 units on day 9. Roots were differentiated on the latter day. Hence, on both the root differentiating media, the peroxidase activity per unit protein was nearly same, being 0.38 units on the former and 0.36 units on the latter.

(c) IAA Oxidase :

The progressive changes of total and specific IAA

Oxidase activity in haploid callus tissue cultured on shoot inducing medium supplemented with various phenolic acids, are presented in Fig. 124-131 and Tables 62-69.

On 100 μM t-cinnamic acid containing medium (a) the total IAA Oxidase activity was on increase right from the beginning till day 9. The specific IAA Oxidase activity increased during the initial 6 days, but declined thereafter till day 9 (Fig. 124, Table 62).

On 500 μM t-cinnamic acid containing medium (b) the total and specific IAA Oxidase activity demonstrated double peaked developmental pattern. The first peak value was reached on day 3 and the second one on day 9 (Fig. 125, Table 63).

The total and specific IAA Oxidase activity in haploid tobacco callus cultured on 100 μM caffeic acid containing medium (c) was on increase right through the culture period of 9 days. The peak activity was attained on day 9 (Fig. 126, Table 64).

With the increase of caffeic acid to 500 μM in the medium (d) the total IAA Oxidase activity did not demonstrate any appreciable increase during the initial 6 days. Thereafter a very sharp increase in the activity ensued and the peak activity reached on day 9. The specific IAA Oxidase activity

declined slightly between days 0 and 3. By day 6 it had increased marginally. Beyond day 6 and till day 9 the activity increased very sharply (Fig. 127, Table 65).

The total and specific IAA Oxidase activity in haploid tobacco callus cultured on 100 μM ferulic acid containing medium (e) demonstrated identical double peaked developmental pattern. The first peak value was reached on day 3 and the second on day 9 (Fig. 128, Table 66).

On 500 μM ferulic acid containing medium (f) the total and specific IAA Oxidase activity reached two peak values during the 9 day culture period. The first peak was reached on day 3 and the second one on day 9 (Fig. 129, Table 67).

The total IAA Oxidase activity was on increase right from the beginning till day 9, on 1.0 μM p-hydroxybenzoic acid containing medium (g). The specific IAA Oxidase activity also followed the same trend (Fig. 130, Table 68).

On 10.0 μM p-hydroxybenzoic acid containing medium (h) though total IAA Oxidase activity was on increase during the initial 6 day, the increase in activity was rather slow. Thereafter, the activity shot up to reach its peak value on day 9. The specific IAA Oxidase activity increased marginally during the initial 3 days of culture. Thereof, the activity increased very rapidly to its peak value by day 9 (Fig. 131, Table 69).

Addition of 100 μM t-cinnamic acid into the shoot forming medium resulted in decline between days 6 and 9 of IAA Oxidase activity per unit protein. All other phenolic acids brought about increase of activity during the corresponding period. On 500 μM t-cinnamic acid containing medium the enzyme activity declined from 0.96 units on day 3 to 0.93 units on day 6 and was followed by root differentiation on day 6. On 100 μM caffeic acid medium the activity increased slightly from 0.93 units on day 6 to 1.04 units on day 9, and was followed by root differentiation on day 9. Hence, on the two media whereon roots differentiated, IAA Oxidase activity per unit protein was 0.93 units and 1.04 units on the day of root differentiation.

(d) MDH :

The progressive changes of total and specific MDH activity in haploid callus cultured on shoot inducing medium supplemented with various phenolic acids, are presented in Figs. 124-131 and Tables 62-69.

The total and specific MDH activity in haploid callus cultured on 100 μM t-cinnamic acid containing medium (a), was on increase throughout the 9 day culture period. The total MDH activity increased rapidly between days 0 and 3. It was followed by a three day period till day 6 when the increase in activity was marginal. Thereof, MDH activity shot up to

attain its peak value on day 9. The specific MDH activity though on increase from day 0 till day 9, demonstrated most rapid increase between days 3 and 6 (Fig. 124, Table 62).

On 500 μM t-cinnamic acid containing medium (b) the increase in total MDH activity was rather modest during the initial 3 days. Thereafter, MDH activity increased rapidly and reached its peak value on day 9. The specific MDH activity declined slightly during the initial 3 days. Thereof, it increased sharply to reach the peak value on day 6. Between days 6 and 9 the activity was on decline (Fig. 125, Table 63).

On 100 μM caffeic acid containing medium (c) total MDH activity increased rapidly between days 0 and 9, reaching peak activity on day 9. The specific MDH activity, however, after rapid increase of activity between days 0 and 6, declined between days 6 and 9 (Fig. 126, Table 64).

The total MDH activity in haploid tobacco callus cultured on 500 μM caffeic acid containing medium (d) increased slightly during the initial 3 days. Thereafter, rapid increase of activity ensued till day 9. The specific MDH activity decreased slightly till day 3. Thereof, the activity increased sharply to reach the peak value on day 9 (Fig. 127, Table 65).

On 100 μM ferulic acid medium (e) the total MDH activity increased slowly between days 0 and 6. It was followed by sharp increase in the activity till day 9. The specific MDH

activity increased between days 0 and 3. By day 6 it had, however, declined. Thereafter the specific MDH activity increased sharply to its peak value on day 9 (Fig. 128, Table 66).

The total and specific MDH activity declined during the initial 3 days on 500 μM ferulic acid containing medium (f). Thereafter, the activity increased sharply to reach the peak value on day 9 (Fig. 129, Table 67).

With the incorporation of 1.0 μM p-hydroxybenzoic acid (g) into the medium, the total and specific MDH activity in haploid tobacco callus declined during the initial 3 days. Thereof, the activity increased sharply to reach the peak value on day 9 (Fig. 130, Table 68).

On 10.0 μM p-hydroxybenzoic acid containing medium (h), the total and specific MDH activity demonstrated double peaked pattern of development. The first peak value was reached on day 3 and the second on day 9 (Fig. 131, Table 69).

With the exception of 500 μM t-cinnamic acid and 100 μM caffeic acid, the addition of all other phenolic acids into the standard shoot inducing medium brought about increase of MDH activity per unit protein between days 6 and 9. On 500 μM t-cinnamic acid medium, whereon roots were differentiated on day 6, the MDH activity per unit protein on that day was 5.63 units. On 100 μM caffeic acid medium, whereon root differentiation occurred on day 9, the MDH activity per unit protein on that day was 5.50 units.

(e) PAL :

The progressive changes of total and specific PAL activity in haploid callus cultured on shoot inducing medium supplemented with various phenolic acids, are illustrated in Figs. 124-131 and Tables 62-69.

Total and specific PAL activity attained peak value on day 6 in haploid callus cultured on 100 μM t-cinnamic acid (a) medium. Thereof, the activity declined very sharply till day 9 (Fig. 124, Table 62).

On 500 μM t-cinnamic acid (b) medium the total PAL activity increased throughout the 9 day culture period, reaching peak value on day 9. The specific PAL activity attained peak value on day 6 and declined thereafter till day 9 (Fig. 125, Table 63).

On 100 μM caffeic acid (c) medium, total PAL activity demonstrated double peaked pattern of development. The first peak value was reached on day 3 and the second on day 9. The specific PAL activity increased sharply between days 0 and 3. Though the activity continued to increase till day 9, however, the rate of increase was slow (Fig. 126, Table 64).

The total PAL activity in haploid tobacco callus cultured on 500 μM caffeic acid (d) medium demonstrated two periods of rapid increase. The first phase was between days 0 and 3, and

the second between days 6 and 9. Between days 6 and 9 the activity increased marginally.The specific PAL activity increased almost linearly and at a rapid rate between days 0 and 9 (Fig. 127, Table 65).

On 100 μM ferulic acid (e) medium the total and specific PAL activity increased sharply between days 0 and 6, to reach the peak value on day 6. Thereafter, the activity declined till day 9 (Fig. 128, Table 66).

A double peaked developmental pattern was exhibited by the total and specific PAL activity in haploid tobacco callus cultured on 500 μM ferulic acid (f) medium. Subsequent to sharp increase in the activity the first peak value was reached on day 3 and the second on day 9 (Fig. 129, Table 67).

On 1.0 μM p-hydroxybenzoic acid (g) medium, the total PAL activity increased steadily and continuously during the entire culture period of 9 days. The specific PAL activity increased sharply between day 0 and 6. Thereafter the activity was on decline till day 9 (Fig. 130, Table 68).

The total PAL activity in haploid tobacco callus cultured on 10.0 μM p-hydroxybenzoic acid (h) medium exhibited double peaked pattern of development. The first peak value was reached on day 3 and the second on day 9. The specific PAL activity increased rapidly between days 0 and 3, reaching the peak value.

Thereof, it was on continuous decline till day 9 (Fig. 131, Table 69).

The PAL activity per unit protein of haploid tobacco callus was on decline between days 6 and 9, when cultured on the shoot inducing medium supplemented with t-cinnamic acid (100, 500 μM), ferulic acid (100 μM) and p-hydroxybenzoic acid (1.0, 10.0 μM). During the corresponding period the activity increased on shoot inducing medium supplemented with caffeic acid (100, 500 μM) and ferulic acid (500 μM). On 500 μM t-cinnamic acid containing medium, whereupon roots were differentiated on day 6, the PAL activity per unit protein increased from 3.39 units on day 3 to 11.28 units on day 6. On 100 μM caffeic acid medium, whereon roots differentiation occurred on day 9, the enzyme activity per unit protein increased from 9.40 units on day 6 to 12.22 units on day 9.

(f) Phenolics :

Progressive changes of the phenolic content in haploid callus cultured on shoot inducing medium supplemented with various phenolic acids, are illustrated in Figs. 124-131 and Tables 62-69.

On 100 μM t-cinnamic acid medium (a) the phenolic content increased from 0.13 mg/culture on day 0 to 1.16 mg/culture on day 6. By day 9 it, however, dropped to 0.71 mg/culture. On

percentage basis the phenolic content increased from 1.12 mg% on day 0 to 2.83 mg% on day 6. By day 9 it had declined to 1.63 mg% (Fig. 124, Table 62).

On culture basis the phenolics accumulated unabated during the 9 day culture period in haploid callus cultured on 500 μM t-cinnamic acid (b) medium. It increased from 0.13 mg/culture on day 0 to 1.82 mg/culture on day 9. On percentage basis the phenolic content increased from 1.12 mg% on day 0 to 3.74 mg% on day 6. By day 9 it declined to 3.53 mg% (Fig. 125, Table 63).

The phenolic accumulation reached its peak on day 9, when haploid callus was cultured on 100 μM caffeic acid containing medium (c). It increased from 0.13 mg/culture on day 0 to 2.49 mg/culture on day 9. On percentage basis the phenolic content increased from 1.12 mg% on day 0 to 3.68 mg% on day 9 (Fig. 126, Table 64).

In haploid callus cultured on 500 μM caffeic acid (d) containing medium, on culture basis phenolic accumulation reached its peak value on day 9. It increased from 0.13 mg/culture on day 0 to 1.25 mg/culture on day 9. On percentage basis the increase was from 1.12 mg% on day 0 to 2.78 mg% on day 9 (Fig. 127, Table 65).

The phenolic content in haploid callus cultured on 100 μM ferulic acid (e) containing medium, increased from 0.13 mg/

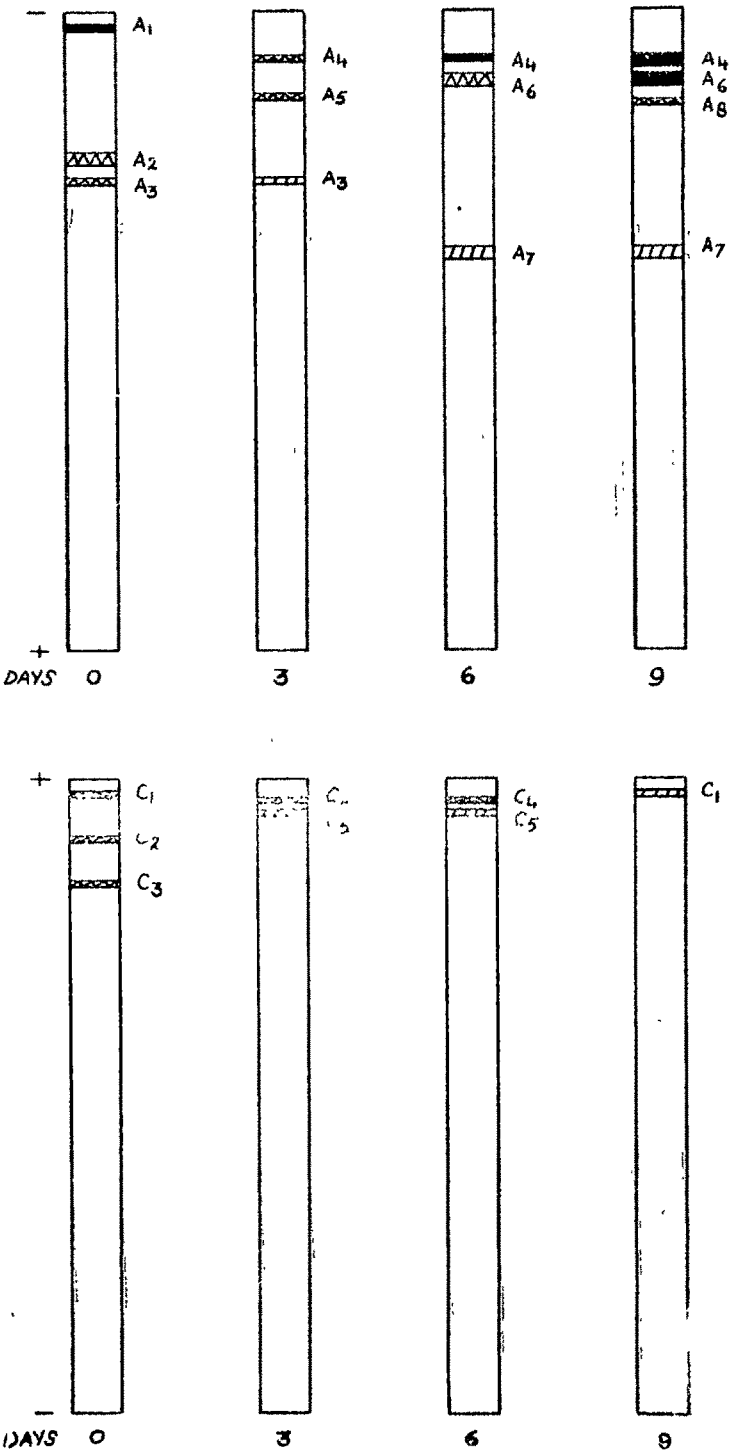
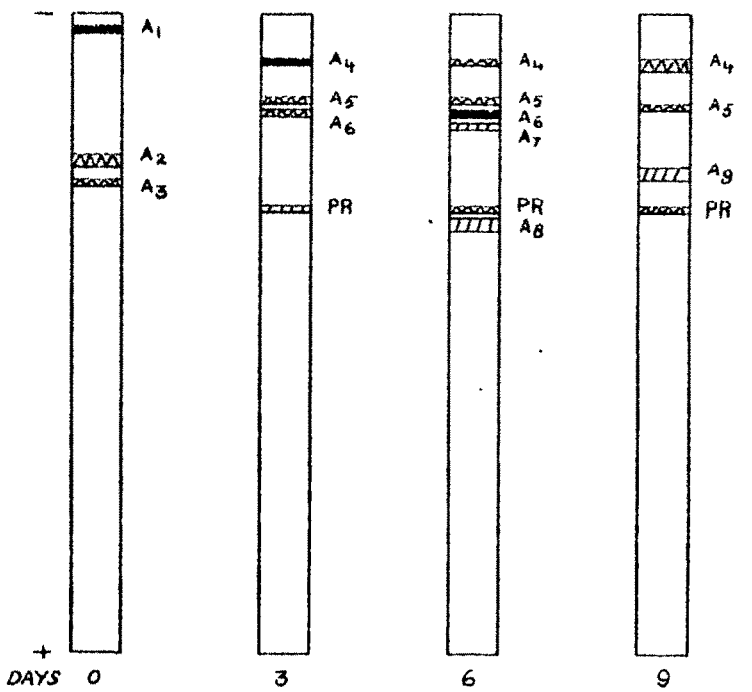


FIG. 124 a..

Anodic and Cathodic Isoperoxidase Patterns.
Experimental Details as in Table 62.



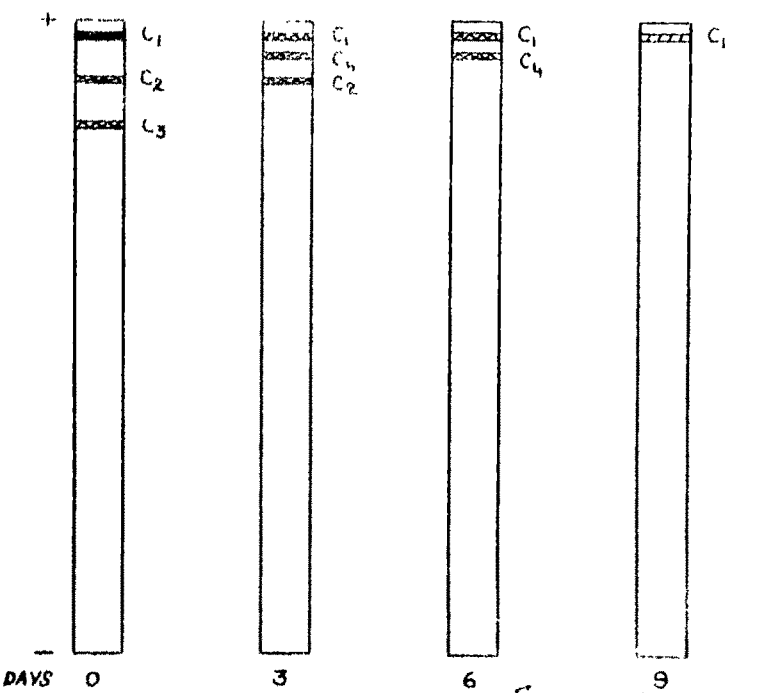
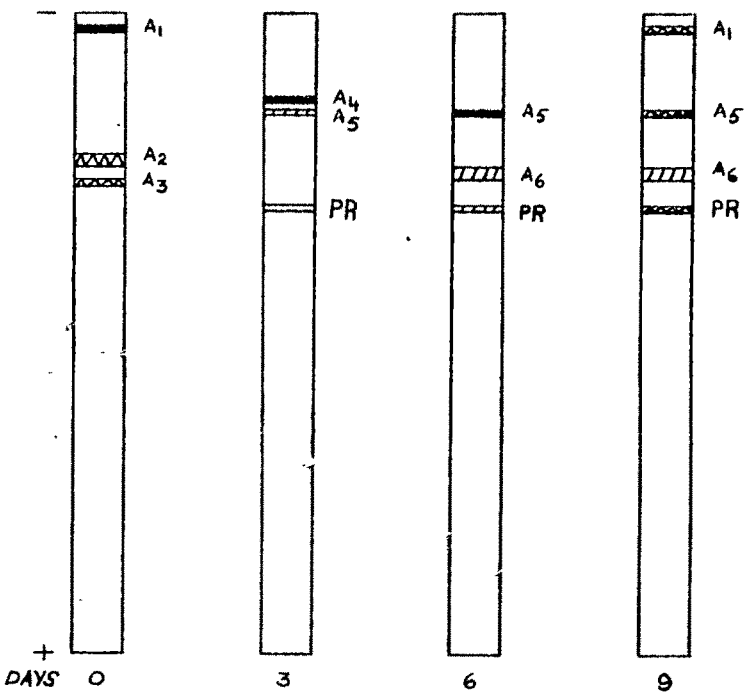


Fig 11.5 a.

Anodic and Cathodic Isoperoxidase Patterns
Experimental Details as in Table 63.



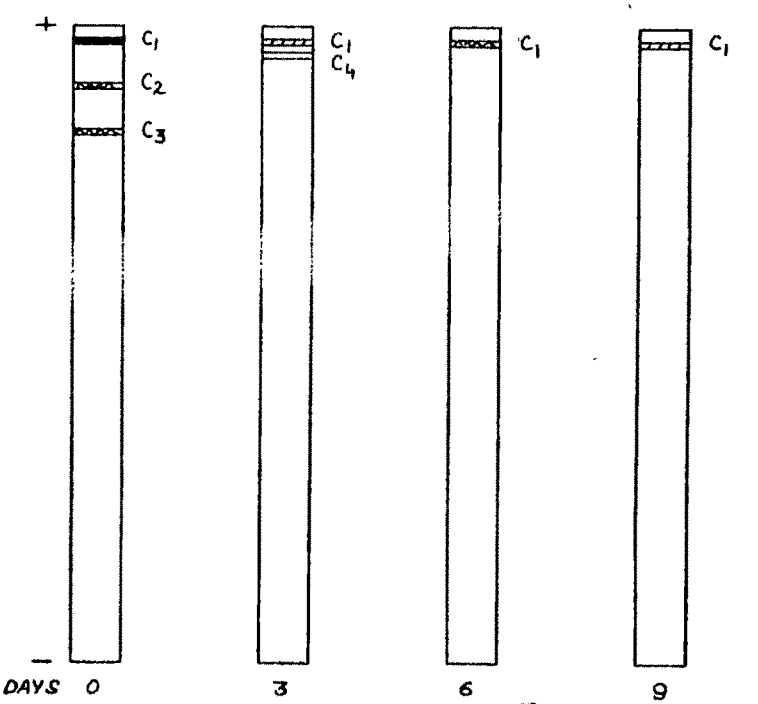


FIG. 126 a.

Anodic and Cathodic Isoperoxidase Patterns.
Experimental Details as in Table 64.

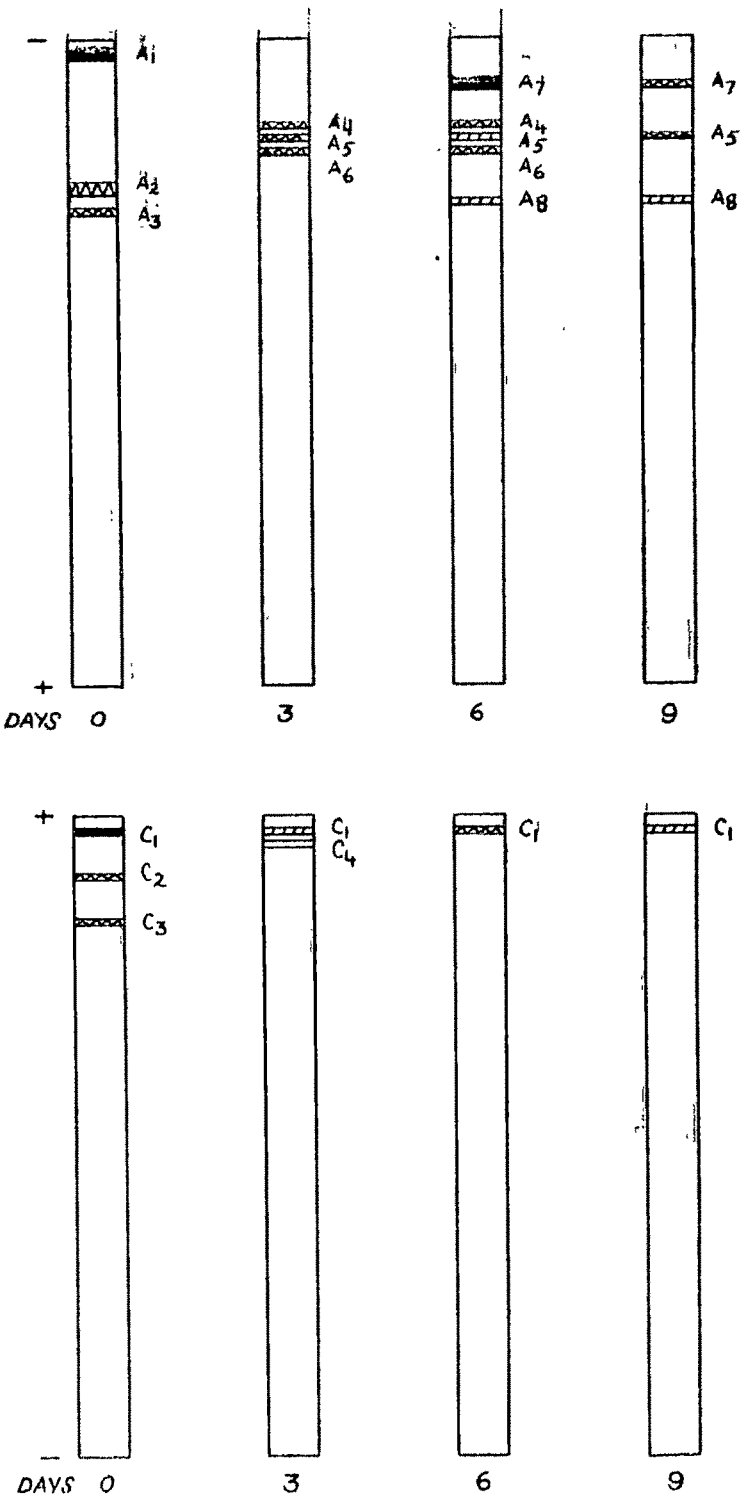


FIG. 127a

Anodic and Cathodic Isoperoxidase Patterns.
Experimental Details as in Table. 65

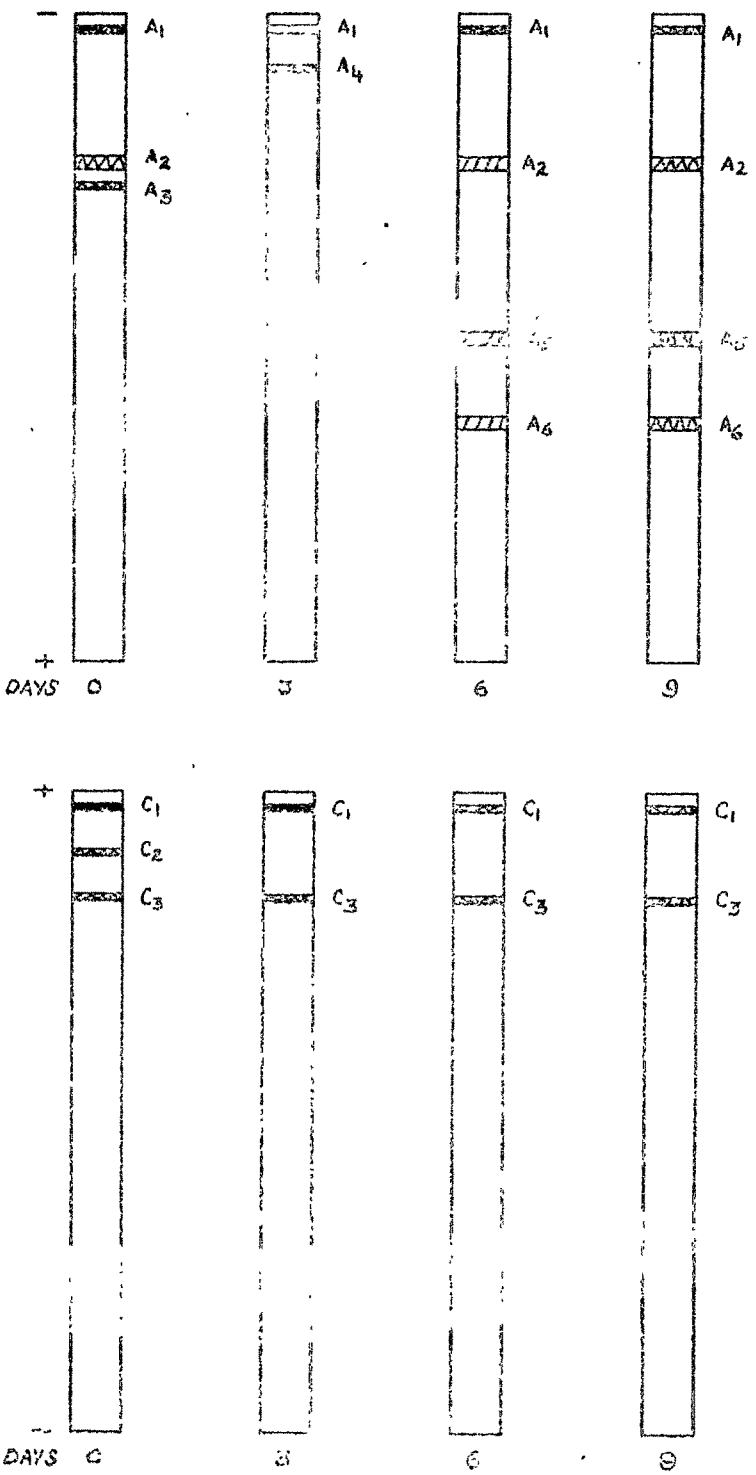
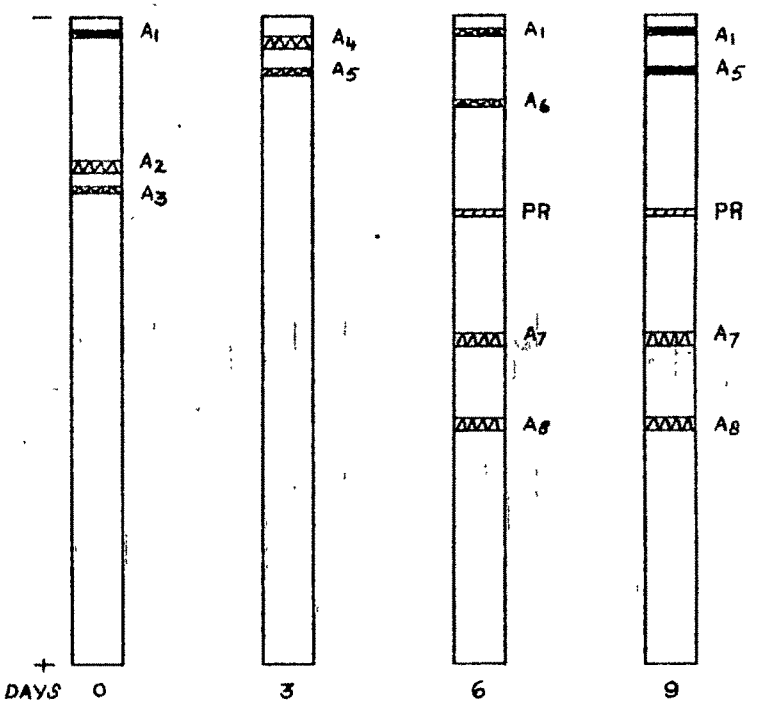


FIG. 12B a

Anodic and Cathodic Isoperoxidase Patterns.
Experimental Details as in Table 66.



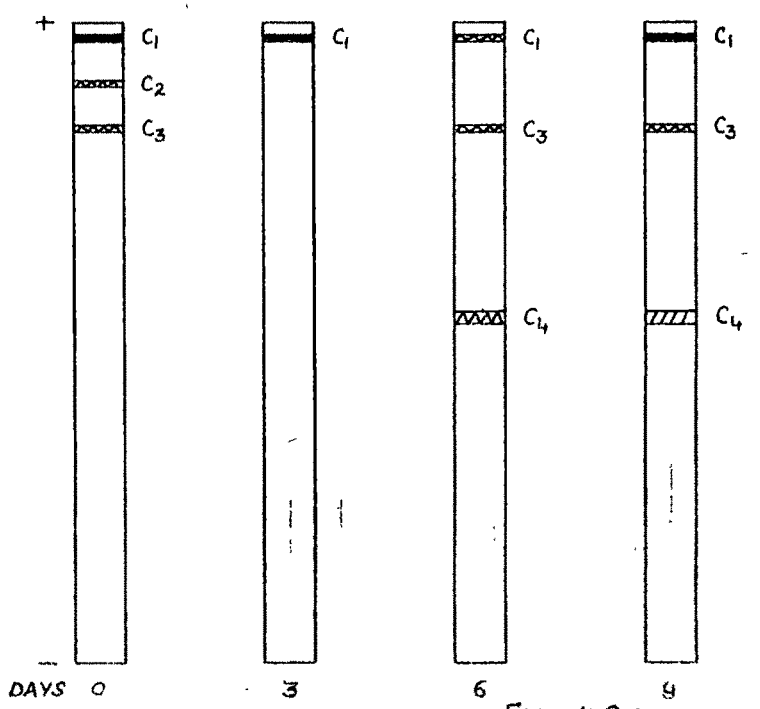
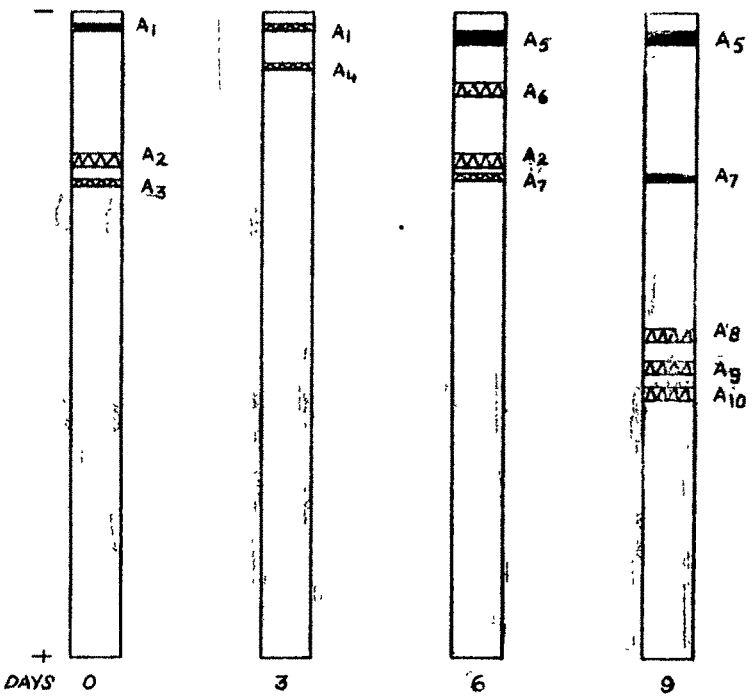


FIG. 129 a.

Anodic and Cathodic Isoperoxidase Patterns.
Experimental Details as in Table 67.



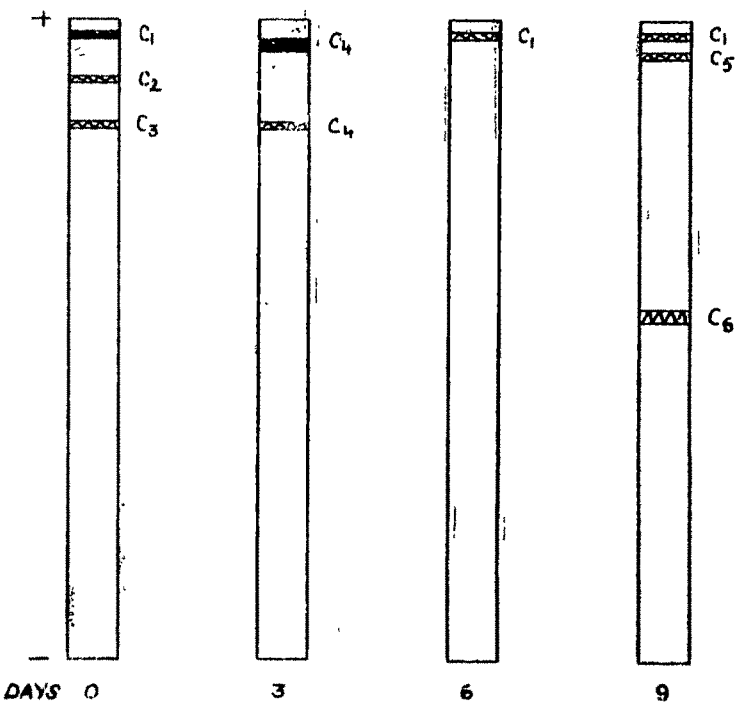
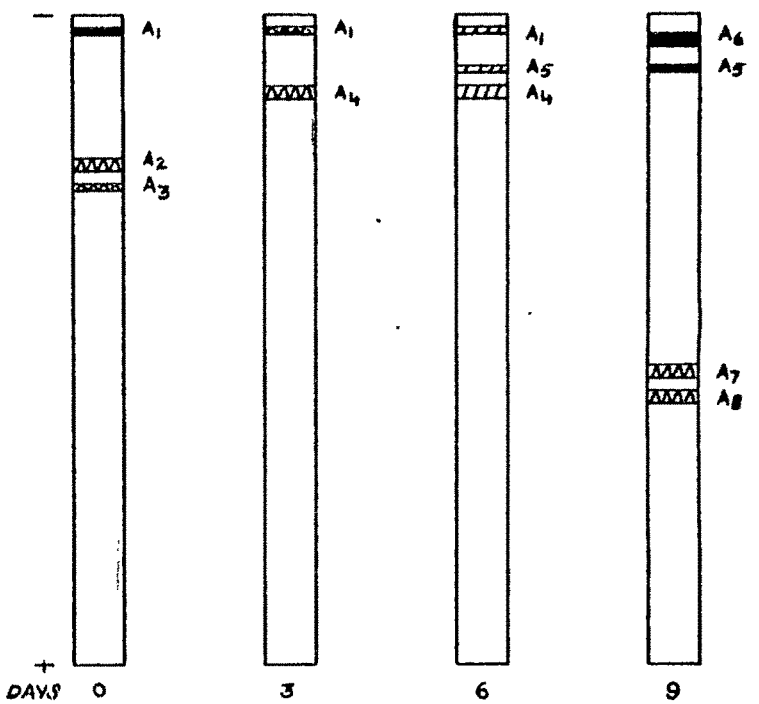


FIG. 130 a.

Anodic and Cathodic Isoperoxidase Patterns.
Experimental Details as in Table 68



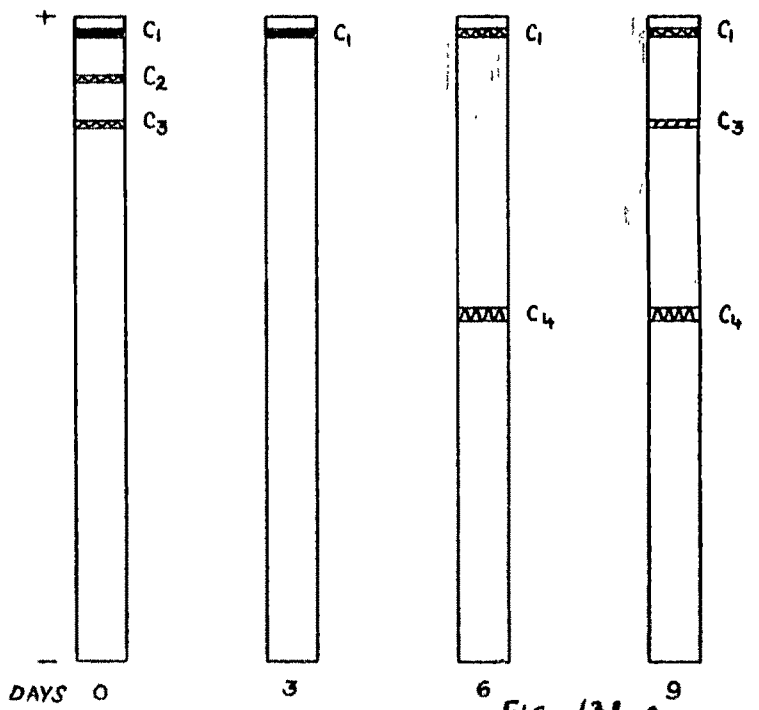


FIG. 131 a.

Anodic and Cathodic Isoperoxidase Patterns.
Experimental Details as in Table 69.

period. A_1, however, appeared on day 9. Three slow migrating anodic isoenzymes of peroxidase A_4 - A_6 were also synthesized. A_4 was synthesized on day 3, A_5 on days 3, 6 and 9, and A_6 on days 6 and 9. The differentiation of roots on this medium on day 9 was preceded by the synthesis of anodic isoperoxidase 'PR' from day 3 onwards. On the cathodic scale the initial isoperoxidases C_2 and C_3 were suppressed for the entire length of culture period. C_1 was, however, synthesized on all days of culture. A slow migrating cathodic isoperoxidase C_4, was synthesized on day 3 (Fig. 126a).

On 500 µM caffeic acid medium (d) all the three initial anodic isoperoxidases A_1, A_2 and A_3 remain suppressed during the entire culture period. However, five slow migrating anodic isoperoxidases A_4 - A_8 were synthesized during the culture period. A_4 and A_6 were synthesized on days 3 and 6, A_5 on days 3, 6 and 9, A_7 and A_8 on days 6 and 9. On the cathodic scale two initial isoperoxidases C_2 and C_3 remain suppressed for the complete culture period. The third initial cathodic isoperoxidase C_1 was synthesized on all days of culture. A slow migrating cathodic isoperoxidase C_4 appeared on day 3 (Fig. 127a).

With the use of 100 µM ferulic acid in the medium (e) the original anodic isoperoxidase A_3 remained suppressed right through the culture period. A_1 was synthesized on all

days and A_2 on days 6 and 9. A slow migrating anodic isoperoxidase A_4 was synthesized on day 3. A_5 and A_6, two fast migrating anodic isoperoxidases were present on days 6 and 9. On the cathodic scale no new isoperoxidases were synthesized. Of the three initial cathodic isoperoxidases, C_1, C_2 and C_3, only one i.e. C_2, was suppressed throughout the culture period (Fig. 128a).

The two original anodic isoperoxidases A_2 and A_3 were suppressed in haploid tobacco callus cultured on 500 μM ferulic acid containing medium (f). The third initial anodic isoperoxidase A_1 was synthesized on days 6 and 9. Three slow migrating anodic isoperoxidases A_4 - A_6 were synthesized during the culture period. A_4 appeared on day 3, A_5 on days 3 and 9, and A_6 on day 6. Two fast migrating ones A_7 and A_8 were synthesized on days 6 and 9. The differentiation of shoots was inhibited on this 500 μM ferulic acid containing medium. Even though there was no visual manifestation of root differentiation, nevertheless, the synthesis of root peroxidase 'PR' on days 6 and 9 ensued. On the cathodic scale the original isoperoxidase C_2 was not synthesized at any time during the culture period. The other original cathodic isoperoxidase C_1 and C_3 were synthesized during the culture period. C_1 was synthesized on days 3, 6 and 9, and C_3 on days 6 and 9. A fast migrating one C_4 was synthesized on days 6 and 9 (Fig. 129a).

Incorporation of 1.0 μM p-hydroxybenzoic acid into the medium (g) resulted in suppression of the original anodic isoperoxidase A_3. Of the other original anodic isoperoxidases, A_1 appeared on day 3 and A_2 on day 6. Four slow migrating anodic isoperoxidases A_4 - A_7 were synthesized. A_4 appeared on day 3, A_5 and A_7 on days 6 and 9, and A_6 on day 6. Three fast migrating anodic isoperoxidases A_8 - A_{10} were synthesized on day 9. On the cathodic scale the original isoperoxidase C_2 remained suppressed, C_1 appeared on days 6 and 9, and C_3 on day 3. On day 3 a slow migrating cathodic isoperoxidase C_4 was synthesized. C_5 a slow migrating and C_6 a fast migrating cathodic isoperoxidases were synthesized on day 9 (Fig. 130a).

The increase of p-hydroxybenzoic acid level to 10.0 μM in the medium (h) resulted in the suppression of original anodic isoperoxidases A_2 and A_3. The third original isoperoxidase A_1, however, appeared on days 3 and 6. Three slow migrating anodic isoperoxidases A_4 - A_6 were synthesized. A_4 was synthesized on days 3 and 6, A_5 on days 6 and 9, and A_6 on day 9. On day 9 two fast migrating isoperoxidases A_7 and A_8 were also synthesized. The initial cathodic isoperoxidase C_2 was suppressed for the entire length of the culture period. C_1 appeared on all days and C_3 on day 9. On days 6 and 9 a fast migrating cathodic isoperoxidase C_4 was synthesized (Fig. 131a).

Expt. 41. Studies with haploid tobacco callus tissue cultured on shoot inducing medium containing ½ X and 2 X Mn^{++} ion concentrations

Healthy, green callus masses of haploid tobacco, weighing 300±30 mg were inoculated on 20 ml of the shoot inducing medium (MS + 0.3 mg/l IAA + 3% sucrose) in which ½ X and 2 X of the optimum Mn^{++} ion levels were used. Standard MS basal medium contained 22.30 mg/l $MnSO_4$, consequently in the media containing ½ X and 2 X Mn^{++} ion levels, 11.15 mg/l and 44.6 mg/l $MnSO_4$ were used respectively.

The culture vessels were incubated at 26±2° in continuous light. Every 3 days, 5 replicates were harvested and analysed for growth, enzymes, peroxidase isoenzyme banding patterns and phenolic accumulation.

(a) Growth :

Growth of haploid callus cultured on the shoot inducing medium containing ½ X and 2 X Mn^{++} ion levels, are illustrated in Figs. 132, 133 and Tables 70, 71.

When Mn^{++} ion level was altered in the medium growth of haploid callus was inhibited in comparison with that on standard shoot inducing medium. On ½ X Mn^{++} ion containing shoot inducing medium the fresh and dry weight increase

during the 15 day culture period was 1.81 and 3.81 fold respectively (Fig. 132, Table 70). On 2 X Mn^{++} ion containing medium the respective fresh and dry weight increase was 1.89 and 3.71 fold during the 15 day culture period (Fig. 133, Table 71). In comparison the fresh and dry weight of haploid tobacco callus cultured on standard shoot inducing medium increased 2.77 and 4.97 fold respectively. Furthermore, these growth values were reached in only 9 days of culture on standard shoot inducing medium.

With deviation from optimum Mn^{++} ion level in the standard shoot inducing medium, the differentiation of shoots was totally inhibited.

(b) Peroxidase :

Progressive changes of total and specific peroxidase activity in haploid callus cultured on shoot inducing medium containing $\frac{1}{2}$ X and 2 X Mn^{++} ion levels, are presented in Figs. 132, 133 and Tables 70, 71.

On $\frac{1}{2}$ X Mn^{++} ion containing medium the total peroxidase activity in haploid callus exhibited double peaked developmental pattern. The first peak was reached on day 6 and the second on day 15. The specific peroxidase activity increased unabated during the first 12 days of culture, reaching its peak value. Thereof, it declined rapidly till day 15

Table : 70. Growth, Polyphenols and progressive changes in the Activity of Peroxidase, IAA Oxidase, MDH and PAL in haploid callus tissues of N. tabacum L. var. Anand-2 cultured on shoot differentiating medium containing $\frac{1}{2}$ x Mn^{++} ion concentration.

Medium : MS (containing $\frac{1}{2}$ x Mn^{++} ion conc.) + 0.3 mg/l IAA + 3% sucrose

Incubation : 15 days at 26±2° in continuous light

Inoculum : 300±30 mg fresh tissue

Day	Fresh weight mg/cult.	Dry weight mg/cult.	PEROXIDASE		IAA OXIDASE		MDH		PAL		PHENOLICS	
			units/ cult.	units/mg protein	units/ cult.	units/mg protein	units/ cult.	units/mg protein	units/ cult.	units/mg protein	mg/ cult.	mg%
0	300 (±30)	12 (±3)	0.98	0.86	0.48	0.42	1.05	0.92	0.28	0.25	0.13	1.12
3	395.0 (±41.3)	21.3 (±2.7)	1.46	1.01	8.09	5.58	2.37	1.63	1.73	1.19	0.39	1.86
6	498.5 (±36.6)	39.8 (±4.7)	2.19	1.79	9.12	7.47	3.58	2.94	19.94	16.34	0.98	2.48
9	500.6 (±48.3)	40.8 (±4.0)	1.80	1.86	12.20	12.58	8.41	8.68	16.71	17.23	0.98	2.39
12	503.2 (±51.6)	41.0 (±2.8)	1.69	2.06	16.09	19.63	10.06	12.27	15.09	18.40	1.01	2.46
15	545.8 (±43.2)	45.7 (±4.9)	2.83	1.24	14.92	6.54	6.54	2.87	17.58	7.71	1.22	2.66

Figures in parenthesis represent standard error.

Data represented is average of 5 replicates.

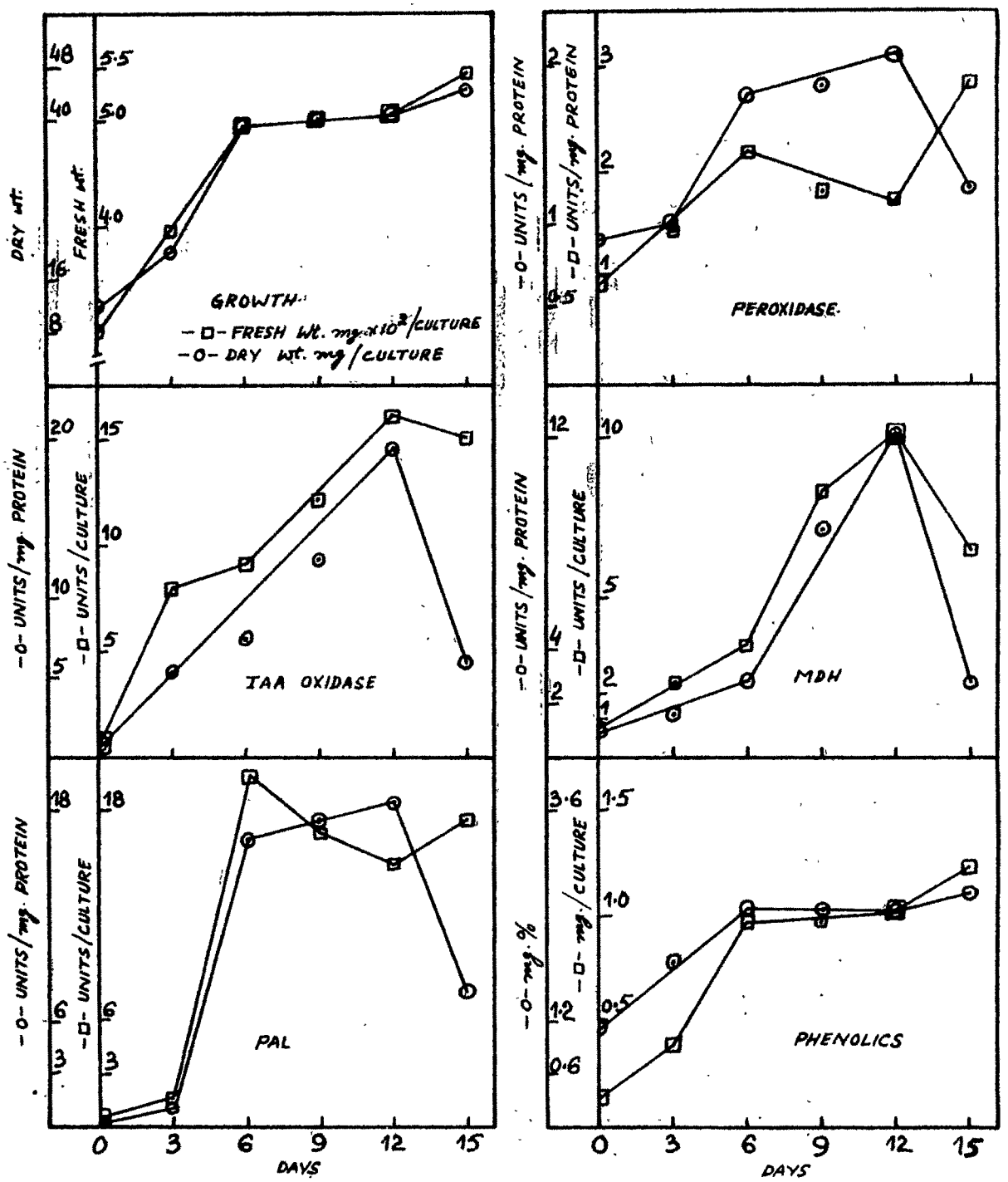


FIG. 132.

Experimental Details as in Table 70.

Table : 71. Growth, Polyphenols and progressive changes in the Activity of Peroxidase, IAA Oxidase, MDH and PAL in haploid callus tissues of *N. tabacum* L. var. Anand-2 cultured on shoot differentiating medium containing 2 x Mn^{++} ion concentration.

Medium : MS (containing 2 x Mn^{++} ion conc.) + 0.3 mg/l IAA + 3% sucrose

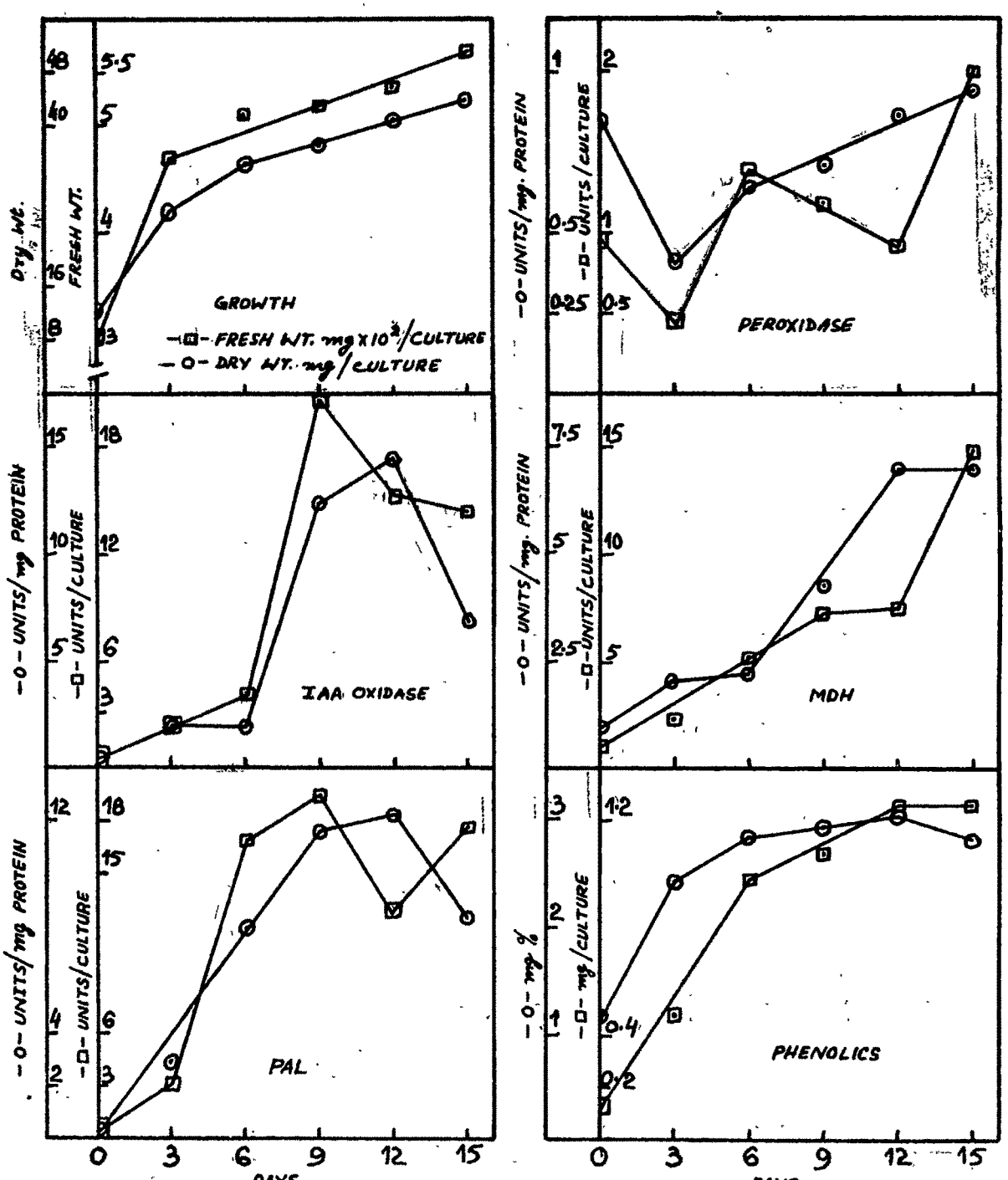
Incubation : 15 days at 26±2° in continuous light

Inoculum : 300±30 mg fresh tissue

Day	Fresh weight mg/cult.	Dry weight mg/cult.	PEROXIDASE		IAA OXIDASE		MDH		PAL		PHENOLICS	
			units/ cult.	units/mg protein	units/ cult.	units/mg protein	units/ cult.	units/mg protein	units/ cult.	units/mg protein	mg cult.	mg%
0	300 (±30)	12 (±3)	0.98	0.86	0.48	0.42	1.05	0.92	0.28	0.25	0.13	1.12
3	469.8 (±41.7)	27.9 (±1.8)	0.43	0.40	1.95	1.80	2.25	2.08	3.12	2.98	0.67	2.42
6	509.3 (±38.2)	34.4 (±3.1)	1.38	0.65	3.99	1.88	4.88	2.30	16.97	8.00	0.98	2.86
9	519.7 (±56.3)	37.1 (±2.9)	1.19	0.71	20.83	12.33	7.23	4.28	19.65	11.63	1.08	2.92
12	534.3 (±48.1)	41.3 (±4.0)	0.91	0.86	15.36	14.49	7.48	7.05	13.05	12.31	1.26	3.06
15	569.4 (±63.6)	44.6 (±4.3)	1.98	0.93	14.49	6.87	14.80	7.01	17.71	8.39	1.26	2.84

Figures in parenthesis represent standard error.

Data represented is average of 5 replicates.



Experimental Details as in Table 71.

FIG. 133.

(Fig. 132, Table 70). On standard shoot inducing medium also, the specific peroxidase activity or peroxidase activity per unit protein was on increase between days 0 and 9. However, the enzyme activity on all days was higher on $\frac{1}{2}$ X Mn^{++} ion containing shoot inducing medium than on the standard shoot forming medium.

The total peroxidase activity in haploid callus cultured on shoot inducing medium containing 2 X Mn^{++} ion level, declined during the initial 3 days of culture. Thereof, it increased to reach its first peak value on day 6. Between days 6 and 12 the peroxidase activity was on decline. However, between days 12 and 15 the activity was on increase again. The specific peroxidase activity declined sharply between days 0 and 3. Thereafter, it was on increase right upto day 15 (Fig. 133, Table 71). The specific peroxidase activity on standard shoot inducing medium was on increase right from day 0 to day 9. Furthermore, the activity on any given day was higher on the standard shoot inducing medium than on the medium containing 2 X Mn^{++} ion concentration.

(c) IAA Oxidase :

Progressive changes of total and specific IAA Oxidase activity in haploid callus cultured on shoot inducing medium containing $\frac{1}{2}$ X and 2 X Mn^{++} ion levels, are illustrated in Figs. 132, 133 and Tables 70, 71.

On ½ X Mn^{++} ion containing medium the total and specific IAA Oxidase activity exhibited identical developmental pattern. The activity increased very rapidly between days 0 and 12, reaching its peak value. Thereof, the activity declined till day 15. The decline of specific activity during last 3 days of culture was substantial (Fig. 132, Table 70). On standard shoot inducing medium the specific IAA Oxidase activity increased rapidly between days 0 and 6, and then declined till day 9. On the other hand, the specific IAA Oxidase activity on ½ X Mn^{++} ion containing medium was 2 to 6 times higher on all days of culture.

On 2 X Mn^{++} ion containing medium the total IAA Oxidase activity increased between days 0 and 9, reaching its peak value. The increase of activity during the initial 6 days was rather modest. Between days 6 and 9 the activity increased very sharply. The activity was on decline between days 9 and 15. The specific IAA Oxidase activity increased, though not very substantially, during the first 6 days of culture. Thereof till day 12, the activity increased sharply to reach the peak value. Equally rapid and sharp was the decline of activity between days 12 and 15 (Fig. 133, Table 71). The Specific IAA Oxidase activity on days 3 and 6 was higher on the standard shoot inducing medium than on the above

mentioned one. On day 9, however, the activity was 4 times more on 2 X Mn^{++} ion containing medium than on standard shoot inducing medium.

(d) MDH :

Illustrated in Figs. 132, 133 and Tables 70, 71 are progressive changes of total and specific MDH activity in haploid callus cultured on shoot inducing medium containing $\frac{1}{2}$ X and 2 X Mn^{++} ion levels.

The developmental pattern of total and specific MDH activity was identical in haploid callus cultured on $\frac{1}{2}$ X Mn^{++} ion containing medium. The MDH activity on both accounts was on increase between days 0 and 12, reaching its peak value. Between days 12 and 15 the activity declined sharply (Fig. 132, Table 70). On the standard shoot inducing medium the specific MDH activity was appreciably higher between days 3 and 9 than on the medium containing $\frac{1}{2}$ X Mn^{++} ion level.

On 2 X Mn^{++} ion containing medium the total MDH activity exhibited a near linear increase during the first 9 days of culture. Between days 9 and 12 the increase of activity was rather insignificant. However, thereafter the total MDH activity increased to reach its peak value on day 15. The specific MDH activity increased rapidly during the first 3 days of culture. Between days 3 and 6 only modest increase

in MDH activity ensued. However, thereof till day 12 the activity increased linearly and sharply to reach the peak value. Between days 12 and 15 the specific MDH activity remained essentially stable (Fig. 133, Table 71). The specific MDH activity on all days was considerably less than on standard shoot inducing medium during the corresponding period.

(e) PAL :

Progressive changes of total and specific PAL activity in haploid callus cultured on shoot inducing medium containing $\frac{1}{2}$ X and 2 X Mn^{++} ion levels, are presented in Figs. 132, 133 and Tables 70, 71.

On $\frac{1}{2}$ X Mn^{++} ion containing medium total PAL activity in haploid callus increased slightly during the initial 3 days of culture. Thereafter the activity increased very sharply to attain the peak value on day 6. Between days 6 and 12 it declined at a slow rate. However, by day 15 it increased again to reach its second peak value. The specific PAL activity demonstrated slight increase during the initial 3 days. It was followed by sharp increase of activity till day 6. The activity continued to increase till day 12, but at a much slower rate than the preceding phase of rapid increase. The peak value was attained on day 12. Between days 12 and 15 the specific PAL activity declined sharply (Fig. 132, Table 70).

In comparison with the standard shoot inducing medium, the PAL activity was 5 to 16 times higher on all days of culture.

The total PAL activity in haploid callus cultured on 2 X Mn^{++} ion containing medium demonstrated double peaked pattern of development. The activity increased rapidly during the initial 9 days to reach the first peak value. The activity was on rapid decline between days 9 and 12. Thereof, the activity increased again till day 15 to achieve the second peak value. The specific PAL activity exhibited rapid increase between days 0 and 12. Between days 12 and 15 the activity was on decline (Fig. 133, Table 71). On all days the specific PAL activity was higher than on standard shoot inducing medium, it being 10 to 50 times higher than on standard shoot inducing medium.

(f) Phenolics :

Progressive changes of phenolic accumulation in haploid callus cultured on shoot inducing medium containing $\frac{1}{2}$ X and 2 X Mn^{++} ion levels, are illustrated in Figs. 132, 133 and Tables 70, 71.

On $\frac{1}{2}$ X Mn^{++} ion containing medium the phenolic content in haploid callus increased from 0.13 mg/culture on day 0

to 0.98 mg/culture on day 6. Between days 6 and 9 the phenolic content remained stable. It was again on increase between days 9 and 15, reaching the peak value of 1.22 mg/culture on day 15. On percentage basis the phenolic content increased from 1.12 mg% to 2.48 mg% during the initial 6 days. By day 9 it declined slightly to 2.39 mg%. Thereafter it increased again till day 15, reaching the value of 2.66 mg% (Fig. 132, Table 70). The phenolic content in haploid tobacco callus on day 6 was 3.45 mg%, and declined on day 9 i.e. the day of shoot differentiation, to 0.96 mg%. On corresponding days on $\frac{1}{2}$ X Mn^{++} ion containing shoot inducing medium the values were 2.48 mg% and 2.39 mg% and the phenolic content continued to increase till day 15.

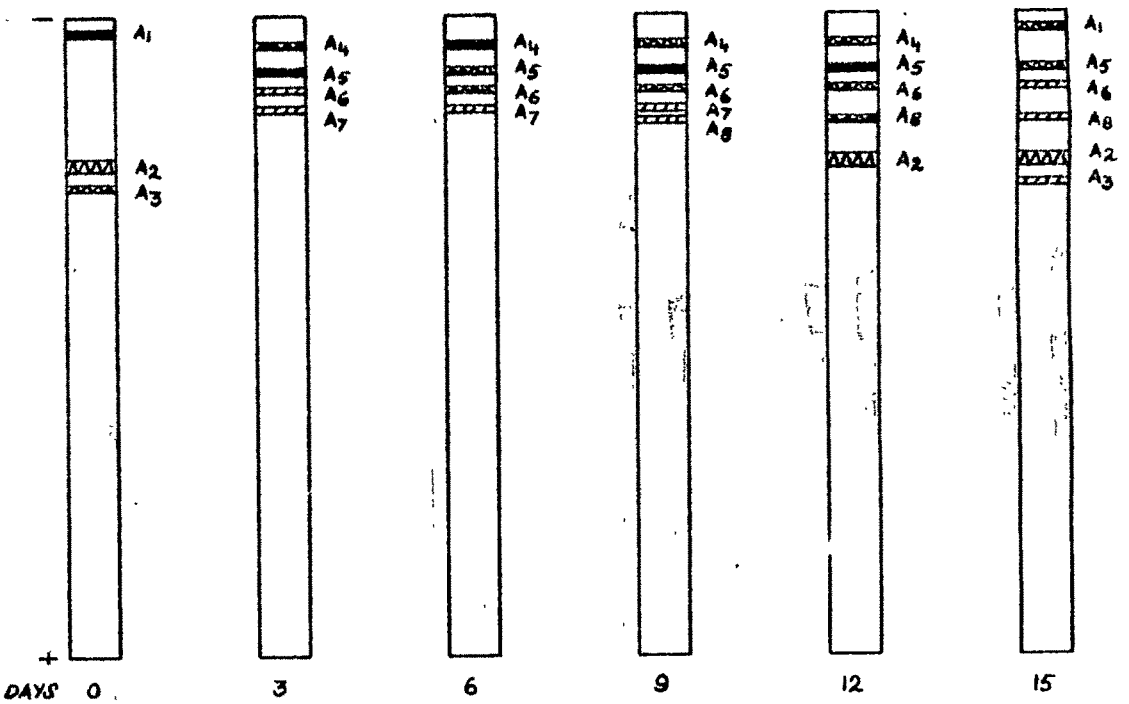
On culture basis the phenolic content in haploid callus cultured on 2 X Mn^{++} ion containing medium increased from 0.13 mg/culture on day 0 to 1.26 mg/culture on day 12. During the subsequent 3 days i.e. till day 15 the value did not change. On percentage basis the peak phenolic accumulation was attained on day 12. During the initial 12 days the phenolic content in haploid tobacco callus increased from 1.12 mg% to 3.06 mg%. By day 15 the phenolic content had declined to 2.84 mg% (Fig. 133, Table 71). On standard shoot inducing medium the phenolic content on day 6 was 3.45 mg% and on day 9 i.e. the day of shoot differentiation, it had declined to 0.96 mg%. On corresponding days the phenolic

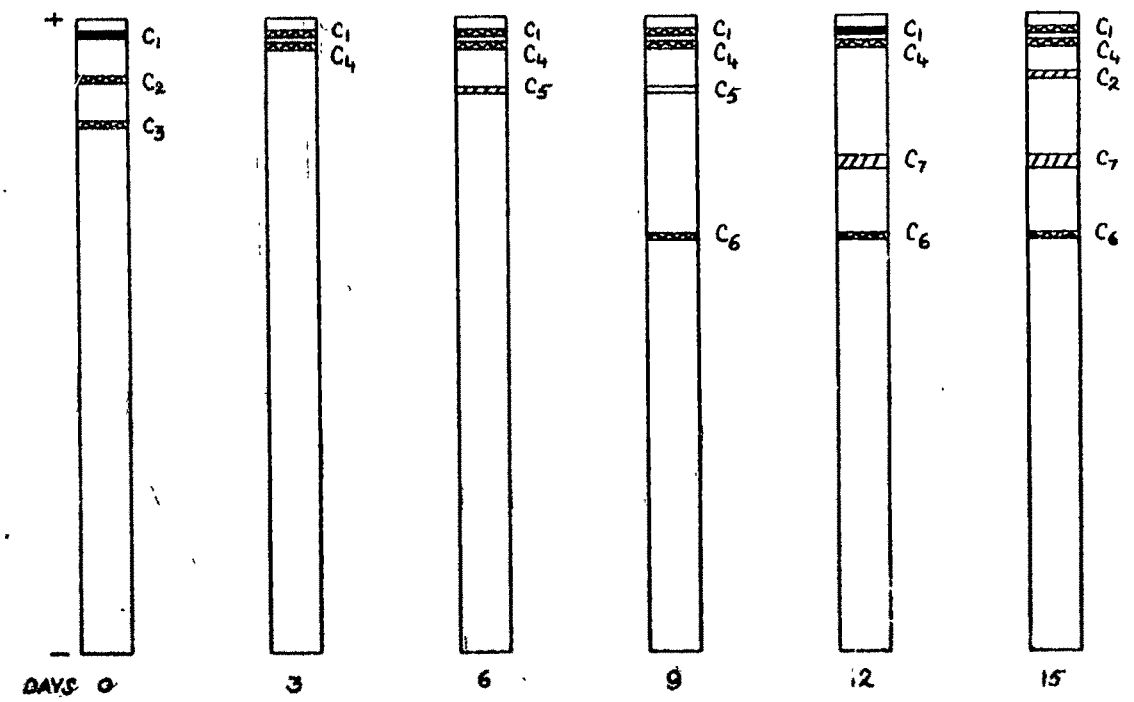
values were 2.86 mg% and 2.92 mg% in haploid tobacco callus cultured on 2 X Mn^{++} ion containing shoot inducing medium.

(g) Peroxidase Isoenzymes :

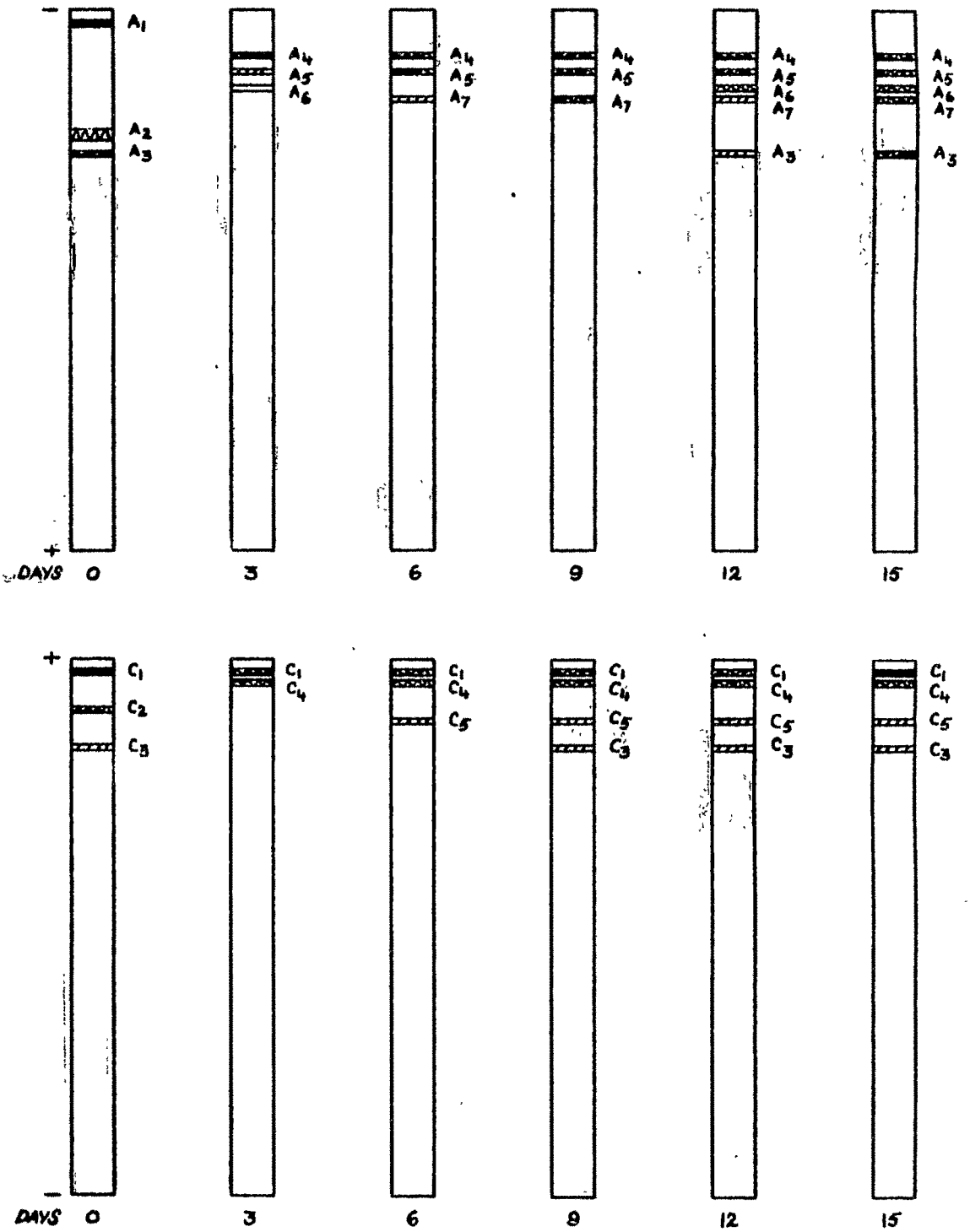
Progressive changes of anodic and cathodic isoperoxidases in haploid callus cultured on shoot inducing medium in which $\frac{1}{2}$ X and 2 X Mn^{++} ion levels were used are presented in Figs. 132a and 133a.

In haploid callus cultured on $\frac{1}{2}$ X Mn^{++} ion containing medium the initial anodic isoperoxidases A_1 and A_3 were suppressed for the entire culture period. The third initial anodic isoperoxidase A_2 was synthesized only on days 12 and 15. During the culture period five slow migrating anodic isoperoxidases A_4 - A_8 were synthesized. A_4 appeared on all days but day 15 of culture, A_5 and A_6 were synthesized on all days of culture, A_7 was synthesized on days 3, 6 and 9, and A_8 on days 9, 12 and 15. On the cathodic side, the initial cathodic isoperoxidase C_3 remained suppressed throughout the culture period. The other original cathodic isoperoxidase C_1 was synthesized on all days and C_2 on day 15 only. Four slow migrating cathodic isoperoxidases C_4 - C_7 were synthesized during the culture period. C_4 was synthesized on all days, C_5 on days 6 and 9, C_6 on days 9, 12 and 15, and C_7 on days 12 and 15 (Fig. 132a).





Anodic and Catodic Esoperoxidase Patterns. FIG. 132 a
Experimentals Details as in Table 70



Anodic and Cathodic Isoperoxidase Patterns. FIG. 133a.
Experimental Details as in Table 71.

On 2 X Mn^{++} ion containing medium the initial anodic isoperoxidases A_1 and A_2 were suppressed throughout the culture period. The third initial anodic isoperoxidase A_3 was synthesized only on days 12 and 15. A_4 - A_7, four slow migrating anodic isoperoxidases were synthesized during the culture period. A_4 and A_5 were synthesized on all days, A_6 on days 3, 12 and 15, and A_7 on days 6, 9, 12 and 15 of culture. On the cathodic side the initial isoperoxidase C_2 was suppressed for the entire culture period, C_3 was synthesized on days 9, 12 and 15, and C_1 on all days of culture. C_4 a slow migrating one was synthesized on all days of culture. C_5, another slow migrating isoperoxidase, was synthesized on all days except day 3 of culture (Fig. 133a).

The differentiation of shoots was inhibited on shoot inducing medium, when the Mn^{++} ion level was altered to either $\frac{1}{2}$ X or 2 X of the optimum concentration. Likewise, the synthesis of anodic isoperoxidases PS_1, PS_2 and PS_3 was inhibited. These isoperoxidases preceded shoot differentiation on standard shoot inducing medium.