CHAPTER III

RESULTS

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Experiments described in this chapter were performed with (1) different parts of plant under different growing periods and (2) tissues initiated from young leaves of Evolvulus alsinoides L. This was done with a view to compare the alkaloid production in intact plant parts and in tissues derived from young leaves. The results obtained with in vivo and in vitro studies are presented in different sections.

SECTION - A

"In vivo" studies on alkaloid production in Evolvulus alsinoides L.

Evolvulus alsinoides (Fig. 6) is an annual herbaceous weed, flowering all the year round except in late summer. The seeds remain in the soil until the rainy season and immediately after rains they germinate into new plants. Alkaloid production/ accumulation was examined in different parts of the plants like : (a) Root, (b) Stem, (c) Leaves, (d) Flowers, and (e) Seeds.

Root, stem and leaves collected from plants at different stages of growth were dried in an oven at 60°C and powdered. Flowers and seeds were also gathered, dried and powdered before analysis. The plants were assayed at the following stages.

FIG. 6. Evolvulus alsinoides L.

(1) Germinated seedlings (2/3 days old), (2) one week old seedlings, (3) young vegetative plant (about 2 weeks old), (4) mature plant during flowering and fruiting (4 to 5 weeks old), and (5) seeds.

In all of the above studies the percentage of alkaloids was very low. Two clear spots of alkaloids with R_f values of 51.66 and 65 were obtained in TLC preparations. Seeds showed the presence of maximum quantity of alkaloids compared to other parts of the plant (Table 3). Young leaves contained very small amount of alkaloid, whereas immediately after germination the seedlings had a fairly good amount of alkaloids. The older leaves as well as the stem from vegetative or flowering stage and flowers did not show the presence of these alkaloids. Roots too contained comparatively smaller amount of alkaloids.

Activity of Tryptophan Synthetase, a key enzyme in ergot alkaloid biogenesis as mentioned in Chapter I, was also studied in germinating seedlings as well as in the leaves of young plants. The enzyme activity was detected in seedling just after germination, while in seedlings one week old and in the leaves, the enzyme activity could not be detected.

Table - 3 : "*In vivo*" studies on alkaloid production and Tryptophan synthetase activity in *Evolvulus alsinoides* L.

Material	Alkaloid (%)/ 100 mg Dry weight	Tryptophan Synthetase units/mg protein
Seedlings (2-3 days old)	0.0006	2.712
Seedlings (1 week old)	0.0005	-
Leaves (Young vegetative plant) (2 weeks old)	0.0004	-
Stem (Young vegetative plant) (2 weeks old)	-	-
Root (2 weeks old vegetative plant)	0.0005	-
Leaves (one month old, Flowering stage)	0.0003	-
Stem (one month old, Flowering stage)	-	-
Root (one month old, Flowering stage)	0.0004	-
Flowers	-	-
Seeds	0.0009	-

SECTION - B

Initiation and establishment of callus from the leaves of E. alsinoides.

Callus was initiated in MS medium containing 2% sucrose and supplemented with 2.0 mg/l, 2,4-D and 0.4 mg/l kinetin (Table 2, Chapter II). Fresh young leaves were cut into small pieces and inoculated in the above medium. Callus development started after 10 days of inoculation. After 30 days the callus masses were subcultured to freshly made medium in order to build up sufficient mass of tissue for further studies (Fig. 7). Callus pieces weighing 300±30 mg by fresh weight were inoculated into each culture flask containing 40 ml agar medium and incubated in continuous light at 25±2°C. The callus tissues were transferred on MS basal medium for about one week, before inoculation onto an experimental medium, to minimise any carry over effects.

FIG. 7. Leaf Callus on completely defined medium.

SECTION - C

C - 1 : Influence of auxins on growth and alkaloid production in *E. alsinoides* callus cultures.

The different auxins studied for their effect on growth and alkaloid production were 2,4-D, NAA, IAA and IBA. Each auxin was used at the concentrations of 0.5, 2.0 and 5.0 mg/l in the MS medium in addition to 2% sucrose, to examine their effect on growth and alkaloid production. The tissue was harvested for the measurement of growth and estimation of alkaloid production after 30 days in culture.

Results showing the effect of 2,4-D concentrations are presented in Table 4 and Fig. 8. Growth as measured by increase in fresh and dry weights, was found to be highest at 2.0 mg/l of 2,4-D. At higher level (5.0 mg/l) of 2,4-D tested, growth was slightly suppressed. Alkaloid production was found to be maximum at 2.0 mg/l of 2,4-D; whereas the lower and higher than 2.0 mg/l concentrations reduced the alkaloid production.

In case of NAA the growth of tissue increased considerably till 5.0 mg/l of NAA. Hence the effect of 10.0 mg/l of NAA was also studied as indicated in Table 5 and Fig. 9. Growth, as measured by increase in fresh and dry weights, was found to be maximum at 5.0 mg/l of NAA. However, at 10.0 mg/l of NAA the growth of tissue appreciably decreased. The alkaloid production

Table - 4. Effect of 2,4-dichlorophenoxy acetic acid on growth and alkaloid production in E. alsinoides callus cultures.

Inoculum : 300 ±30 mg tissue by fresh weight (dry weight 16.08 mg) in 40 ml MS medium containing 2% sucrose and supplemented with 0.5, 2.0 or 5.0 mg/l 2,4-D.

Incubation : 4 weeks at 25 ±2°C in continuous light.

2,4-D (mg/l)	Fresh weight (mg/cult.)	Dry weight (mg/cult.)	Alkaloid	
			(%)	(µg/cult.)
0.5	3490.15 (±27.071)	161.86 (± 7.553)	0.002	3.2
2.0	3517.18 (± 22.129)	163.66 (±4.479)	0.0045	7.4
5.0	3304.86 (±19.538)	150.43 (±6.404)	0.002	3.01

Data represents average of five replicates.

Figures in parenthesis represent standard error.

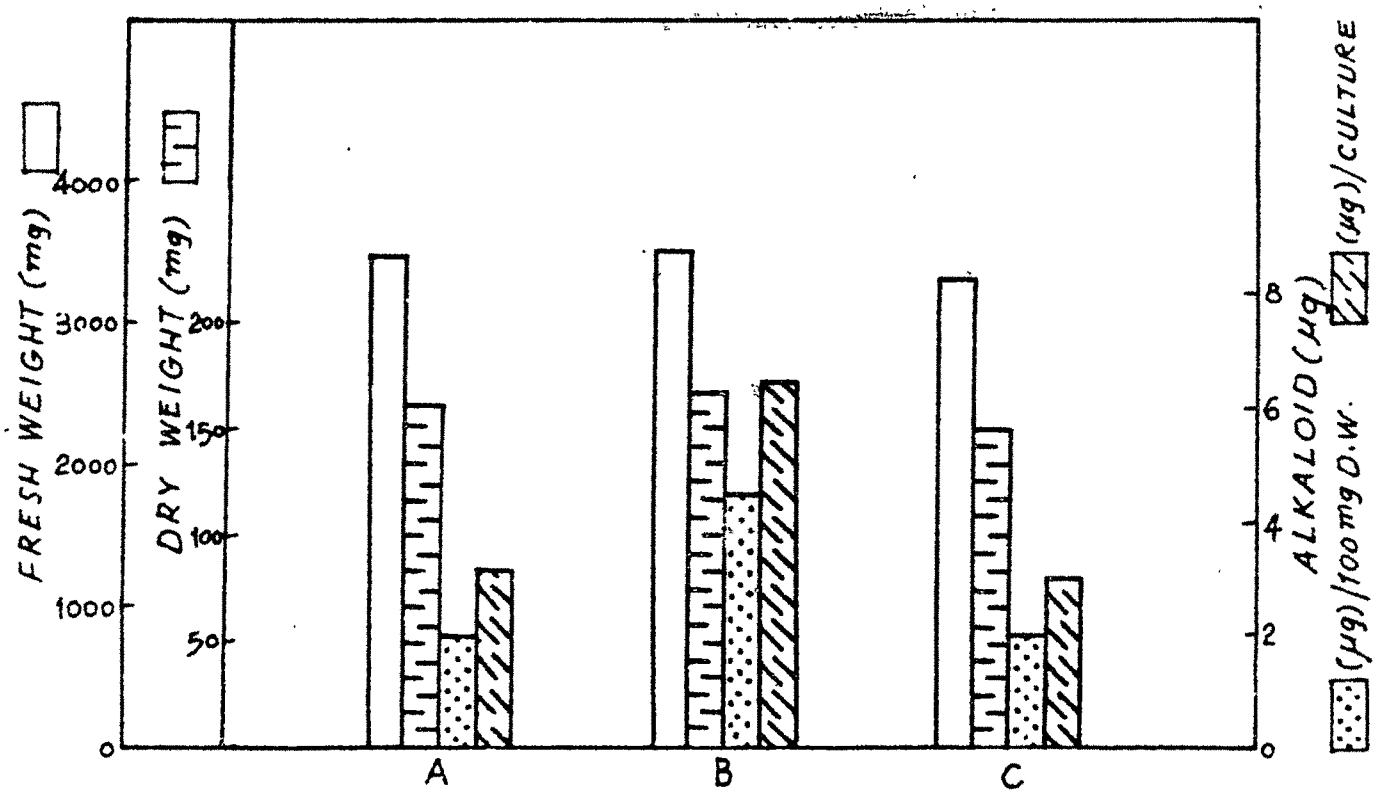

FIG. 8 - EFFECT OF 2,4-D ON GROWTH AND ALKALOID PRODUCTION

[A - 0·5 mg/l 2,4-D B - 2·0 mg/l 2,4-D C - 5·0 mg/l 2,4-D]

Table - 5. Effect of Naphthalene acetic acid on growth and alkaloid production in E. alsinoides callus cultures.

Inoculum : 300 ±30 mg tissue by fresh weight (dry weight 16.08 mg) in 40 ml of MS medium containing 2% sucrose and supplemented with 0.5, 2.0, 5.0 or 10.0 mg/l NAA.

Incubation : 4 weeks at 25 ±2°C in continuous light.

NAA (mg/l)	Fresh weight (mg/cult.)	Dry weight (mg/cult.)	Alkaloid (%)	Alkaloid (µg/cult.)
0.5	3239.8 (±18.776)	191.4 (±4.404)	0.00225	4.31
2.0	3225.64 (±18.612)	182.56 (±5.596)	0.00325	5.9
5.0	3545.1 (±22.481)	173.84 (±6.541)	0.004	6.95
10.0	2854.32 (±16.231)	162.12 (±7.231)	0.00301	4.83

Data represents average of five replicates.

Figures in parenthesis represent standard error.

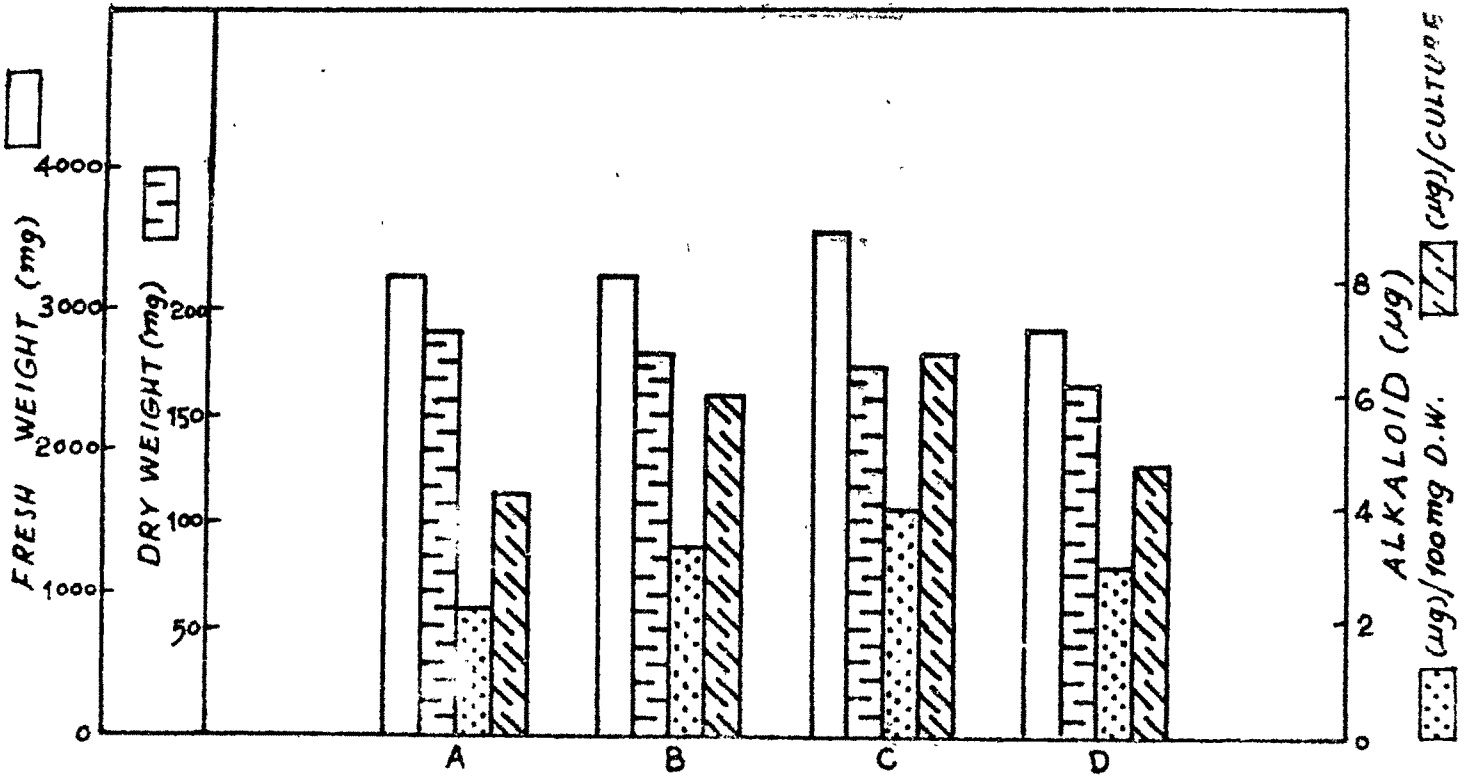

FIG. 9 - EFFECT OF NAA ON GROWTH AND ALKALOID PRODUCTION

[A - 0·5 mg/l NAA B - 2·0 mg/l NAA C - 5·0 mg/l NAA D - 10·0 mg/l NAA]

steadily increased till 5.0 mg/l of NAA, but further increase of NAA resulted in less production of alkaloid.

The results presented in Table 6 and Fig. 10, clearly indicate that IAA shows maximum growth of tissue in fresh weight and dry weight at 5.0 mg/l, the highest level of IAA tested. Alkaloid production was considerably less when compared to the other auxins. The maximum production of alkaloid was recorded at 2.0 mg/l of IAA. Hence concentrations of IAA higher than 5.0 mg/l were not studied even though the growth of tissue was found to be increasing till 5.0 mg/l IAA.

Results showing the effect of IBA concentrations are presented in Table 7 and Fig. 11. Growth as measured by increase in fresh and dry weights was found to be highest at 0.5 mg/l of IBA. Increasing the level of IBA had an adverse effect on growth. Alkaloid production was also found to be maximum at 0.5 mg/l IBA; whereas higher levels of IBA reduced the alkaloid production.

C - 2 : Effect of Cytokinin on growth and alkaloid production in Evolvulus callus cultures.

Measured quantities of tissue weighing 300±30 mg, grown in auxin-cytokinin free medium for one week, were transferred to 40 ml of MS medium supplemented with 0.01, 0.04, 0.4 and 2.0 mg/l of kinetin in addition to 2% sucrose, to find out the effect of kinetin on growth and alkaloid production. Results

Table - 6. Effect of Indole acetic acid (IAA) on growth and alkaloid production in E. alsinoides callus cultures.

Inoculum : 300 ±30 mg tissue by fresh weight (dry weight 16.08 mg) in 40 ml of MS medium containing 2% sucrose and supplemented with 0.5, 2.0 or 5.0 mg/l IAA.

Incubation : 4 weeks at 25 ±2°C in continuous light.

IAA (mg/l)	Fresh weight (mg/cult.)	Dry weight (mg/cult.)	Alkaloid (%)	Alkaloid (µg/cult.)
0.5	3520.9 (±14.314)	188.9 (±3.412)	0.001	1.89
2.0	3916.3 (±15.214)	195.9 (±3.971)	0.002	3.91
5.0	4125.3 (±14.443)	227.1 (±5.923)	0.001	2.27

Data represents average of five replicates.

Figures in parenthesis represent standard error.

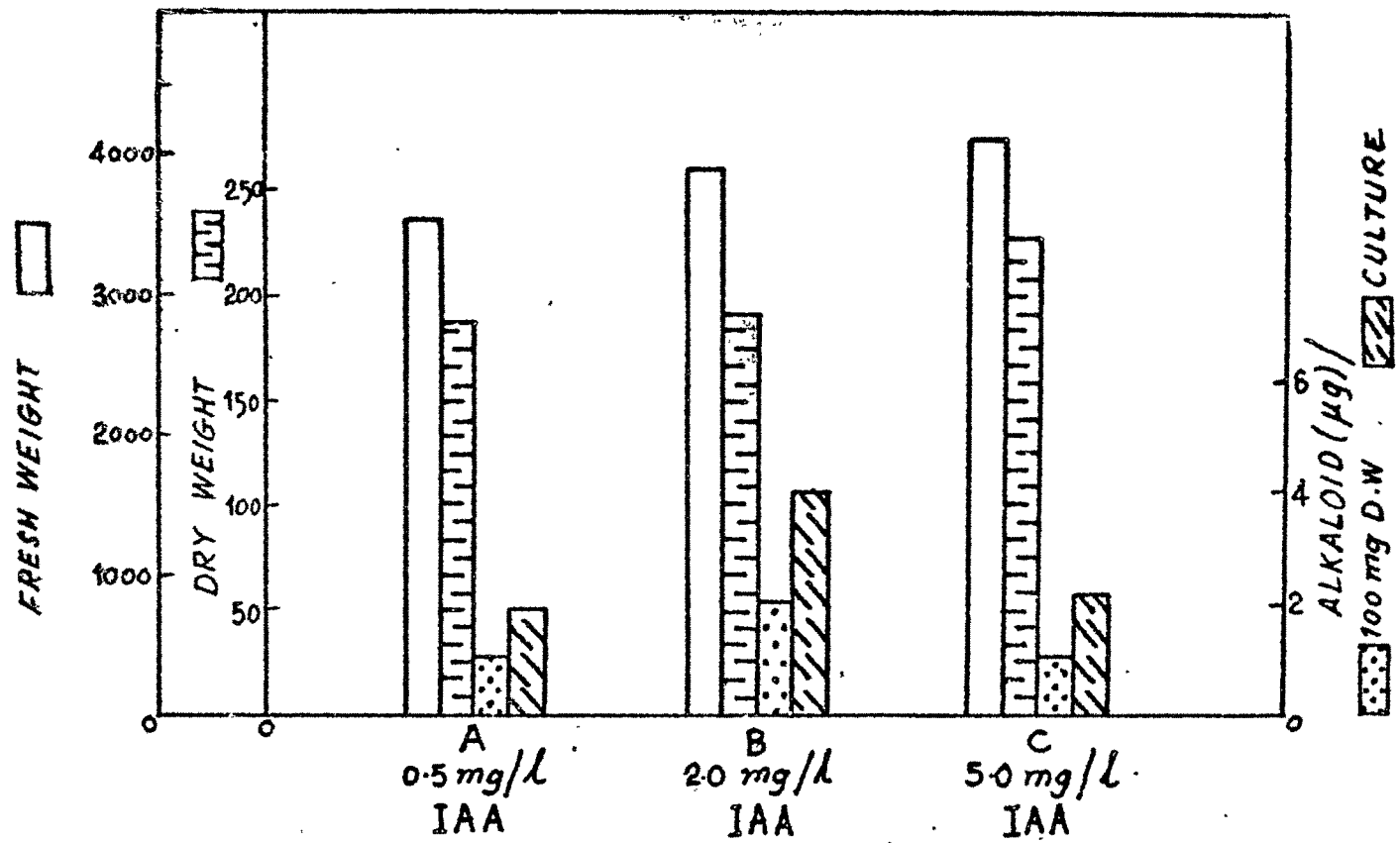

FIG. 10. EFFECT OF IAA ON GROWTH & ALKALOID PRODUCTION

Table - 7. Effect of Indole-3-butyric acid (IBA) on growth and alkaloid production in E. alsinoides callus cultures.

Inoculum : 300 ±30 mg tissue by fresh weight (dry weight 16.08 mg) in 40 ml of MS medium containing 2% sucrose and supplemented with 0.5, 2.0 or 5.0 mg/l IBA.

Incubation : 4 weeks at 25 ±2°C in continuous light.

IBA (mg/l)	Fresh weight (mg/cult.)	Dry weight (mg/cult.)	Alkaloid (%)	Alkaloid (µg/cult.)
0.5	3983.64 (±23.591)	206.6 (±7.733)	0.004	8.3
2.0	3020.1 (±16.316)	172.66 (±5.635)	0.003	5.18
5.0	3065.4 (±28.388)	172.71 (±5.141)	0.002	3.5

Data represents average of five replicates.

Figures in parenthesis represent standard error.

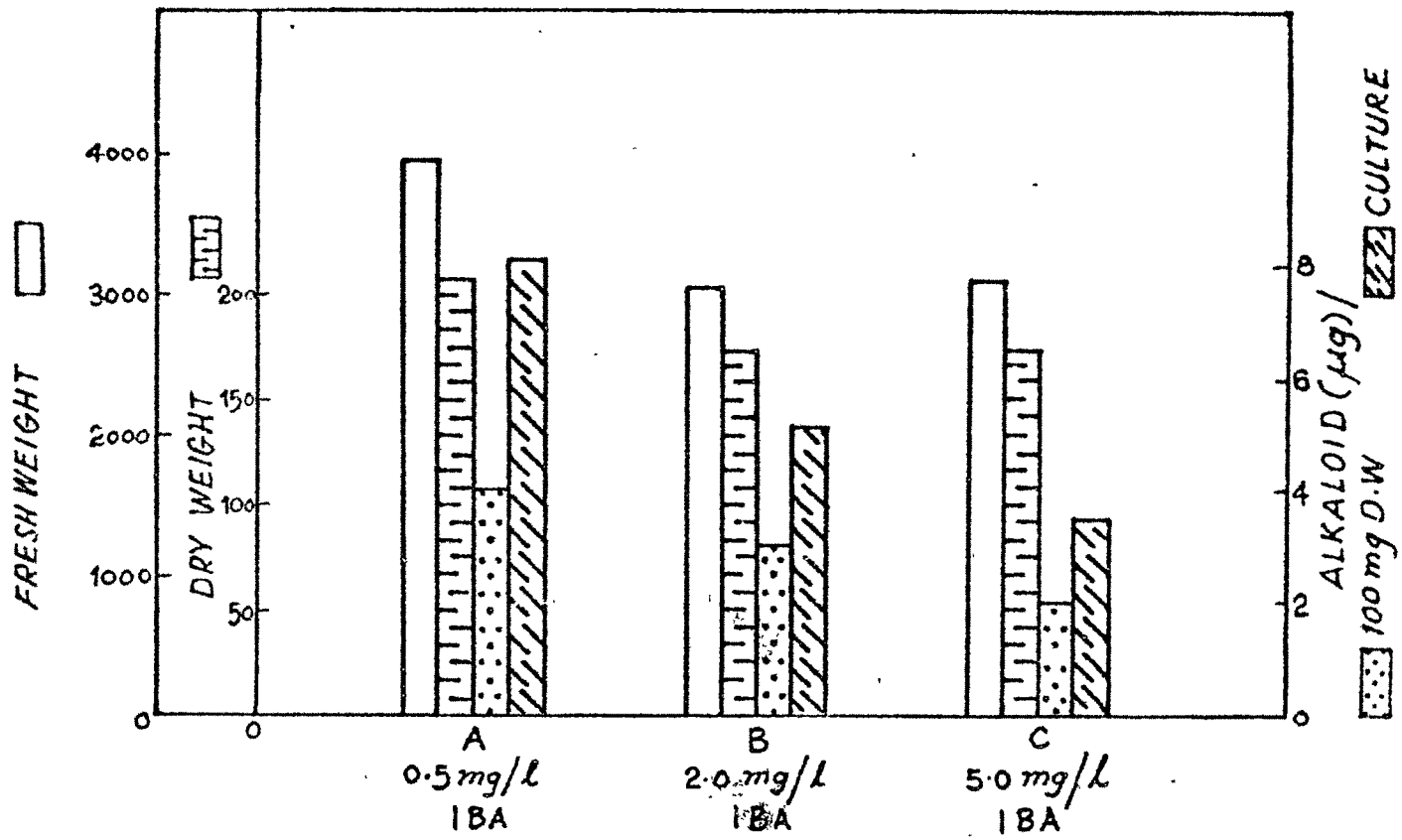

FIG. 11. EFFECT OF IBA ON GROWTH & ALKALOID PRODUCTION

are presented in Table 8 and Fig. 12. Growth was found to be maximum at 0.4 mg/l kinetin. At higher levels of kinetin tested, growth of tissue was considerably reduced. Alkaloid production reached its peak at 0.4 mg/l of kinetin and remained steady at high kinetin level also.

C - 3: Effect of Gibberellic acid (GA_3) on growth and alkaloid production in Evolvulus callus cultures.

Measured quantities of tissue weighing 300±30 mg grown in auxin-cytokinin free medium were transferred to 40 ml of MS medium supplemented with 10, 50 or 100 mg/l GA_3 in addition to 2% sucrose. The tissue was harvested after 30 days for measurement of growth and alkaloid production. Results showing the effect of GA_3 concentrations are presented in Table 9 and Fig. 13.

Growth was found to be more at the lowest concentration of GA_3 tested, whereas it steadily decreased and showed considerably less growth at the highest GA_3 level tested. Alkaloid production was also more at 10 mg/l of GA_3, whereas it remained steady at 50 and 100 mg/l of GA_3.

C - 4 : Effect of Auxin, Cytokinin combinations on growth and alkaloid production in Evolvulus callus cultures.

To examine the effect of auxin and cytokinin in combinations, the auxin concentrations supporting maximum growth of tissue and

Table - 8. Effect of kinetin (K) on growth and alkaloid production in E. alsinoides callus cultures.

Inoculum : 300 ±30 mg tissue by fresh weight (dry weight 16.08 mg) in 40 ml of MS medium containing 2% sucrose and supplemented with 0.01, 0.04, 0.4 or 2.0 mg/l K.

Incubation : 4 weeks at 25 ±2°C in continuous light.

K (mg/l)	Fresh weight (mg/cult.)	Dry weight (mg/cult.)	Alkaloid (%)	Alkaloid (µg/cult.)
0.01	3142.3 (± 7.261)	188.4 (±2.34)	0.002	3.8
0.04	4286.3 (±9.385)	195.8 (±2.26)	0.002	3.9
0.4	4459.1 (±6.323)	198.5 (±1.531)	0.003	5.95
2.0	3200.0 (±11.231)	188.5 (±1.256)	0.003	5.7

Data represents average of five replicates.

Figures in parenthesis represent standard error.

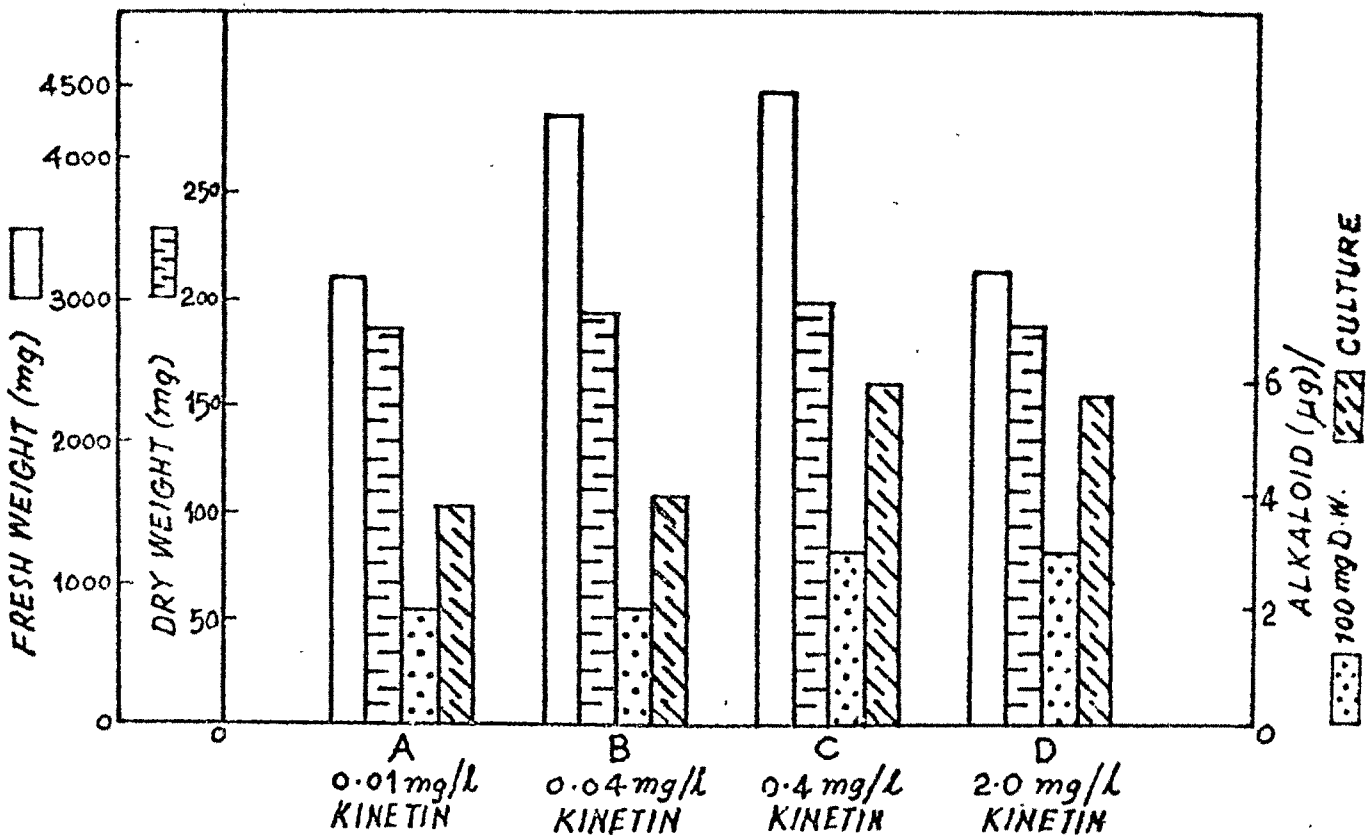

FIG. 12. EFFECT OF KINETIN ON GROWTH & ALKALOID PRODUCTION

Table - 9. Effect of Gibberellic acid (GA_3) on growth and alkaloid production in E. alsinoides callus cultures.

Inoculum : 300 ±30 mg tissue by fresh weight (dry weight 16.08 mg) in 40 ml of MS medium containing 2% sucrose and supplemented with 10, 50 or 100 mg/l GA_3.

Incubation : 4 weeks at 25 ±2°C in continuous light.

GA_3 (mg/l)	Fresh weight (mg/cult.)	Dry weight (mg/cult.)	Alkaloid	
			(%)	(µg/cult.)
10	3091.6 (± 17.098)	159.7 (± 3.456)	0.0035	5.59
50	2996.6 (± 16.064)	149.4 (± 3.399)	0.002	2.99
100	2418.3 (± 10.46)	144.2 (± 1.372)	0.002	2.88

Data represents average of five replicates.

Figures in parenthesis represent standard error.

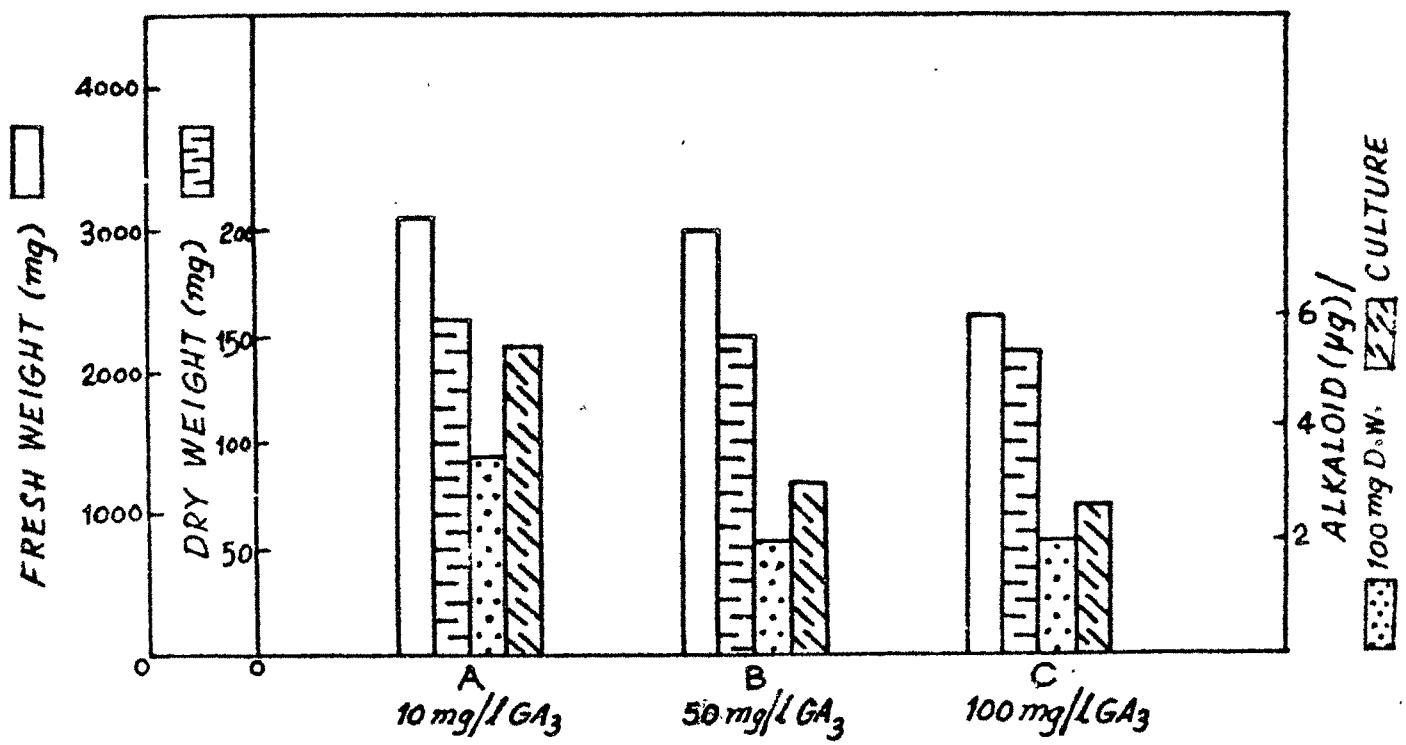

FIG. 13. EFFECT OF GA_3 ON GROWTH & ALKALOID PRODUCTION

alkaloid production was added along with kinetin concentrations which gave higher growth and alkaloid production. The results obtained are presented in Table 10 and Fig. 14. The influence of GA_3 and kinetin was also studied to find out their optimal levels for maximum growth and alkaloid production.

Among the auxins or GA_3 and kinetin combinations tried, MS medium with 2 mg/l 2,4-D and 0.4 mg/l kinetin showed maximum growth of tissue and alkaloid production (Fig. 7). Hence MS medium supplemented with 2 mg/l 2,4-D and 0.4 mg/l kinetin in addition to 2% sucrose was used for further experiments.

C - 5 : Periodical changes in growth and alkaloid production in MS medium supplemented with optimal hormonal concentrations in Evolvulus static and suspension cultures.

300±30 mg tissue was transferred to 40 ml of MS basal medium containing 2% sucrose and supplemented with 2.0 mg/l 2,4-D and 0.4 mg/l kinetin - an optimal combination found in the previous experiment. The tissues were harvested after every 5 days interval till 45 days for determining growth of tissue and alkaloid production. Periodic analysis was carried out in static as well as suspension cultures to compare the growth and alkaloid production in both the systems.

The results of periodic study on growth and alkaloid production in static cultures are presented in Table 11 and

Table - 10. Effect of growth substances on growth and alkaloid production in E. alsinoides callus cultures.

Inoculum : 300±30 mg tissue by fresh weight (dry weight 18.08 mg) in 40 ml of MS medium containing 2% sucrose and supplemented with -
(A) 2 mg/l 2,4-D + 0.4 mg/l K
(B) 5 mg/l NAA + 0.4 mg/l K
(C) 2 mg/l IBA + 0.4 mg/l K
(D) 2 mg/l IBA + 0.4 mg/l K
(E) 10 mg/l GA_3 + 0.4 mg/l K

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

Medium	Fresh weight (mg/cult.)	Dry weight (mg/cult.)	Alkaloid (%)	Alkaloid (µg/cult.)
A	3619.4 (±13.434)	135.0 (±2.032)	0.005	6.9
B	2135.6 (±15.027)	139.3 (±4.529)	0.004	5.6
C	1634.7 (± 6.158)	138.3 (±4.347)	0.0035	4.8
D	1348.1 (± 3.251)	128.6 (±1.039)	0.002	2.6
E	1922.4 (± 6.611)	137.0 (±1.033)	0.004	5.5

Data represents average of five replicates.

Figures in parenthesis represent standard error.

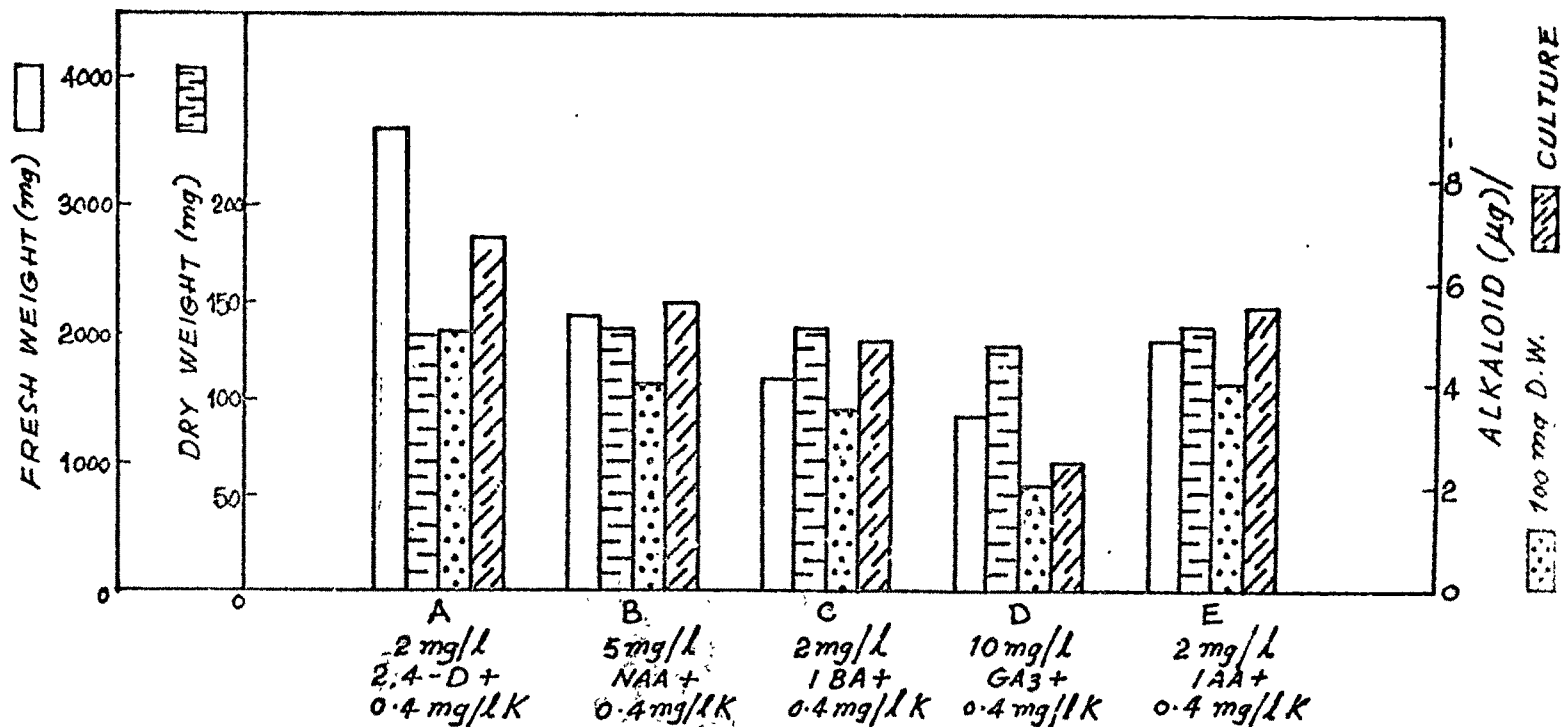

FIG. 14 - EFFECT OF AUXIN/GA_3+KINETIN LEVELS ON GROWTH & ALKALOID PRODUCTION

Table - 11. Periodic changes in growth and alkaloid production in E. alsinoides callus and suspension cultures in a completely defined medium.

Inoculum : (A) 300±30 mg tissue by fresh weight (dry weight 18.08 mg) in 40 ml of MS medium containing 2% sucrose and supplemented with 2.0 mg/l 2,4-D and 0.4 mg/l kinetin.
(B) 200±10 mg tissue by fresh weight (dry weight 11.08 mg) in 40 ml of MS medium containing 2% sucrose and supplemented with 2.0 mg/l 2,4-D and 0.4 mg/l kinetin.

Incubation : 45 days at 25±2°C in continuous light.

	Callus Cultures (A)				Suspension Cultures (B)			
Days	Fresh weight (mg/cult.)	Dry weight (mg/cult.)	Alkaloid (%)	Alkaloid (µg/cult.)	Fresh weight (mg/cult.)	Dry weight (mg/cult.)	Alkaloid (%)	Alkaloid (µg/cult.)
0	300.00 (±1.517)	18.08 (±0.175)	0.004	-	200.00 (±1.232)	11.08 (±0.125)	0.011	-
5	476.64 (±3.858)	25.0 (±1.017)	0.002	0.5	631.94 (±4.714)	41.94 (±1.131)	0.006	2.52
10	819.3 (±1.253)	44.8 (±1.018)	0.002	0.896	1108.56 (±2.198)	71.72 (±1.491)	0.006	4.3
15	1458.1 (±5.489)	70.86 (±1.098)	0.003	2.1	1473.1 (±3.881)	97.04 (±1.968)	0.009	8.73
20	3251.8 (±7.521)	139.3 (±1.123)	0.004	5.6	2280.2 (±9.301)	135.1 (±1.326)	0.01	13.5
25	4054.1 (±10.921)	157.6 (±1.163)	0.005	7.9	4092.6 (±14.761)	198.8 (±3.528)	0.012	23.856
30	4026.0 (±10.131)	155.0 (±1.632)	0.004	6.2	4084.6 (±29.846)	197.9 (±3.701)	0.01	19.8
45	4006.0 (± 9.621)	150.0 (±1.931)	0.002	3.0	-	-	-	-

Data represents average of five replicates.

Figures in parenthesis represent standard error.

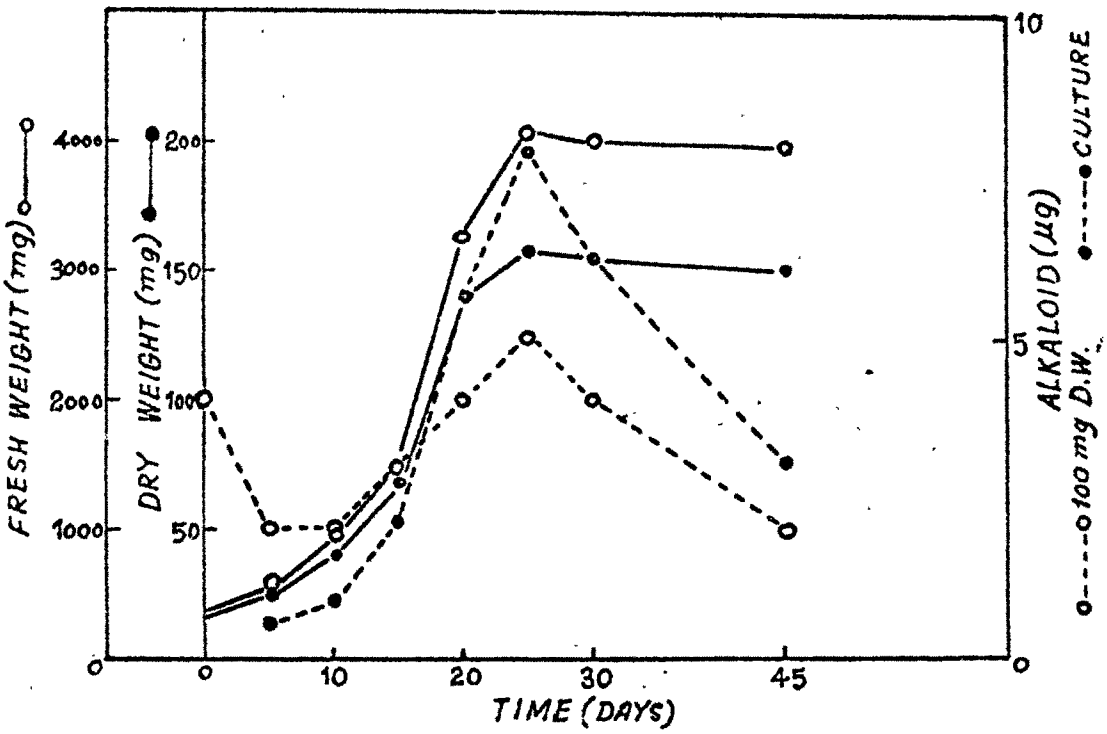

FIG.15. GROWTH & ALKALOID PRODUCTION IN CALLUS CULTURES

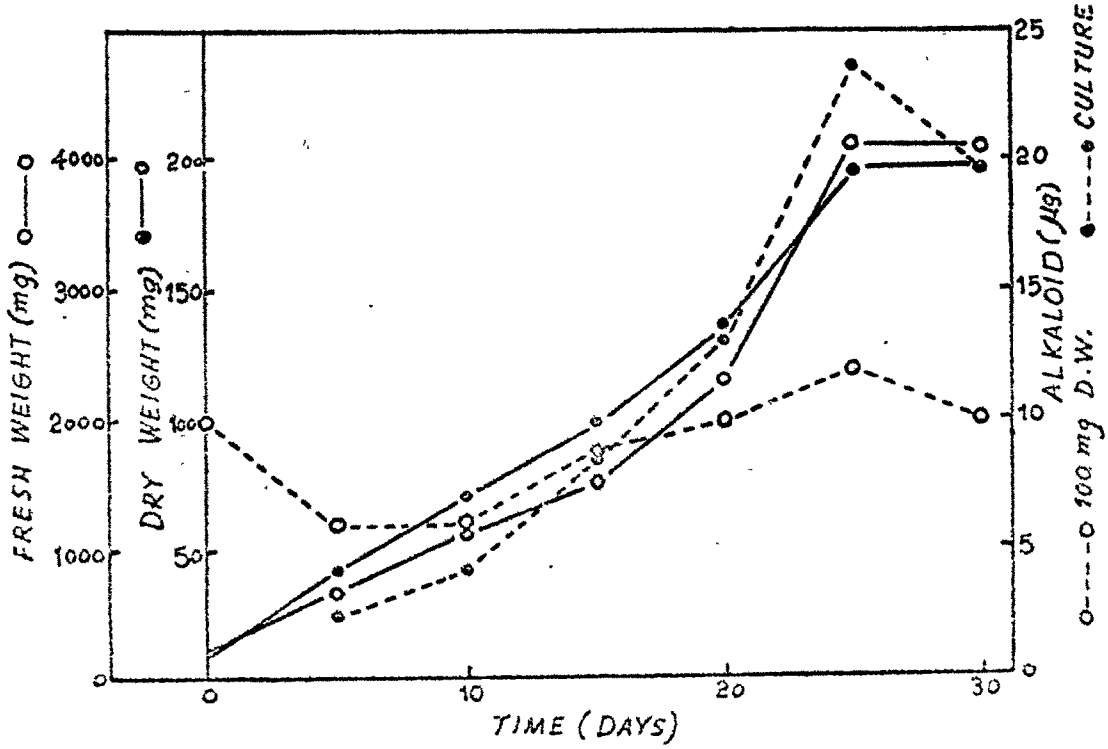

FIG.16. GROWTH & ALKALOID PRODUCTION IN SUSPENSION CULTURES

Fig. 15. The fresh weight and dry weight of the tissue steadily increased from day 5 of culture till day 25. Highest growth of tissue was recorded on day 25 and then it declined slightly till day 45. The tissue showed about 13 fold increase in fresh weight on day 25 and about 9 fold increase in dry weight. Alkaloid production did not increase till day 15, after which it steadily increased until day 25; but then it declined showing the lowest on day 45.

The increase in fresh weight and dry weight was considerably more in suspension cultures compared to static cultures as shown in Table 11 and Fig. 16. To study the growth of tissue and alkaloid production in suspension cultures, 2 ml of cell suspension (200±20 mg in fresh weight) were transferred into 150 ml Erlenmeyer flasks containing 40 ml of MS medium supplemented with 2 mg/l 2,4-D and 0.4 mg/l kinetin in addition to 2% sucrose. The flasks were incubated on a horizontal rotary shaker in continuous light at 25±2°C.

The growth steadily increased recording maximum growth on day 25. The fresh weight increase was 20 fold and dry weight increase was 19 fold as recorded on day 25. The alkaloid production remained steady till day 10 and then it increased steadily, day 25 recording the maximum production of alkaloids. Suspension cultures clearly showed considerably more growth of tissue and alkaloid production compared to the static cultures.

C - 6 : Tryptophan Synthetase activity in Evolvulus static and suspension cultures.

For enzyme assay a fixed number of replicates was harvested at the intervals of 5 days. The tissue was pooled out and assayed for tryptophan synthetase activity as described in Materials and Methods (Chapter II). Specific activity of tryptophan synthetase in static cultures expressed per milligram protein is shown in Table 12 and Fig. 17.

Enzyme activity was nil till day 5. Activity could be detected on day 10 and then it steadily increased till day 20, when the maximum activity was recorded. The enzyme activity then declined recording no activity on day 30. A similar pattern was observed in suspension cultures as shown in Table 12 and Fig. 18. As in the case of growth of tissue and alkaloid production, the tryptophan synthetase activity was also observed to be more in suspension cultures than in static cultures. Hence, all subsequent experiments were carried out in suspension cultures.

Table - 12. Periodic changes in growth and tryptophan synthetase activity in *E. alsinoides* callus and suspension cultures in a completely defined medium.

Inoculum : (A) 300±30 mg tissue by fresh weight (dry weight 18.08 mg) in 40 ml of MS medium containing 2% sucrose and supplemented with 2.0 mg/l 2,4-D and 0.4 mg/l kinetin.

(B) 200±10 mg tissue by fresh weight (dry weight 11.08 mg) in 40 ml of MS medium containing 2% sucrose and supplemented with 2.0 mg/l 2,4-D and 0.4 mg/l kinetin.

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

Days	Callus Cultures (A)			Suspension Cultures (B)		
	Fresh weight (mg/cult.)	Tryptophan Synthetase (units/mg protein)	Tryptophan Synthetase (units/cult.)	Fresh weight (mg/cult.)	Tryptophan Synthetase (units/mg protein)	Tryptophan Synthetase (units/cult.)
0	300.00 (±1.517)	-	-	200.00 (±1.232)	-	-
5	476.64 (±3.858)	-	-	631.94 (±4.714)	-	-
10	819.3 (± 1.253)	14.629	60.24	1108.56 (±2.198)	22.228	66.48
15	1458.1 (± 5.489)	40.416	164.678	1473.1 (±3.881)	27.586	147.30
20	3251.8 (± 7.521)	31.36	162.042	2280.2 (±9.3010)	45.906	199.5
25	4054.1 (±10.921)	25.95	150.676	4092.6 (±14.761)	42.934	171.732
30	4026.0 (±10.131)	-	-	4084.6 (±29.846)	-	-

Data represents average of five replicates.

Figures in parenthesis represent standard error.

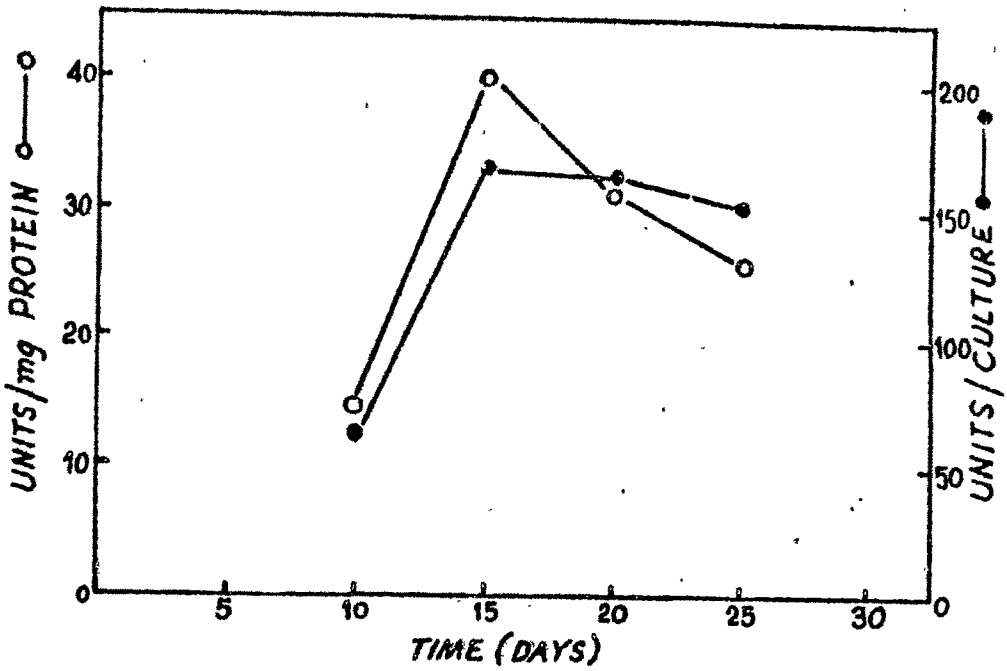

FIG. 17. TRYPTOPHAN SYNTHETASE ACTIVITY IN CALLUS CULTURES

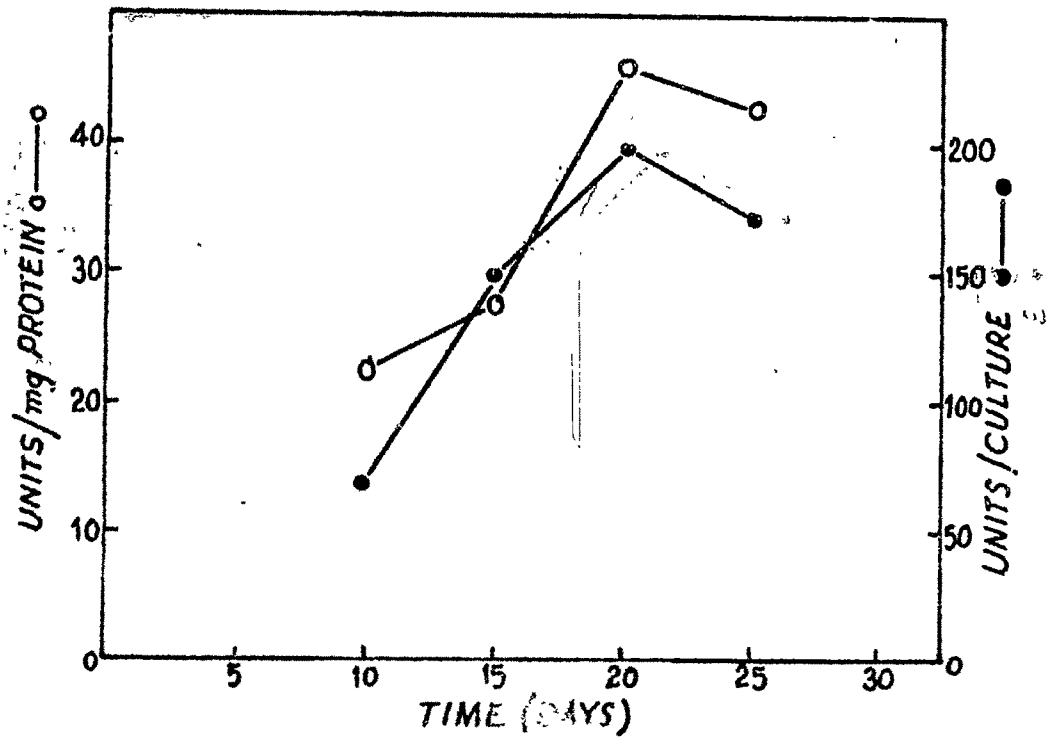

FIG. 18. TRYPTOPHAN SYNTHETASE ACTIVITY IN SUSPENSION CULTURES

SECTION - D

Influence of Inoculum : Volume ratio on growth and alkaloid production in *E. alsinoides* suspension cultures.

To find out the optimal size of inoculum as well as volume of medium which can support maximum growth of tissue and alkaloid production, different sizes of inoculumi in relation to quantity of medium were examined. The different combinations of inoculum size and quantity of medium tested were :

(A) 100 mg inoculum in 25 ml of medium
(B) 100 mg inoculum in 40 ml of medium
(C) 200 mg inoculum in 25 ml of medium
(D) 200 mg inoculum in 40 ml of medium
(E) 300 mg inoculum in 25 ml of medium
(F) 300 mg inoculum in 40 ml of medium.

Among these combinations tried, 200 mg of inoculum in 25 ml of MS medium supplemented with 2 mg/l 2,4-D and 0.4 mg/l kinetin in addition to 2% sucrose showed highest growth of tissue as well as alkaloid production (Table 13, Fig. 19). Alkaloid content percentage-wise was about the same when 200 mg tissue was inoculated in 40 ml of medium. However, growth by fresh and dry weights being higher in case of 200 mg tissue inoculation in 25 ml of medium, the total yield of alkaloid at the end of culture period of 25 days was higher in this treatment. Further, as it saved 15 ml of medium also, this combination of inoculum and volume was preferred for subsequent experiments.

Table - 13. Effect of inoculum size and volume of medium on growth and alkaloid production in E. alsinoides suspension cultures.

Inoculum : 100, 200 or 300 mg tissue by fresh weight in 25 ml, or 40 ml of MS medium containing 2% sucrose and supplemented with 2.0 mg/l 2,4-D and 0.4 mg/l kinetin.

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

Inoculum size	Quantity of medium	Fresh weight (mg/cult.)	Dry weight (mg/cult.)	Alkaloid	
				(%)	(µg/cult.)
100 mg	25 ml	3200.01 (±16.251)	140.01 (±7.232)	0.01	14.0
100 mg	40 ml	2800.7 (±14.671)	135.2 (±6.215)	0.01	13.5
200 mg	25 ml	4087.06 (±17.832)	198.01 (±7.161)	0.012	23.76
200 mg	40 ml	3700.12 (±15.561)	165.72 (±5.261)	0.012	19.886
300 mg	25 ml	2172.13 (±13.121)	129.13 (±5.165)	0.009	11.6
300 mg	40 ml	3912.78 (±15.621)	185.23 (±7.814)	0.011	20.375

Data represents average of five replicates.

Figures in parenthesis represent standard error.

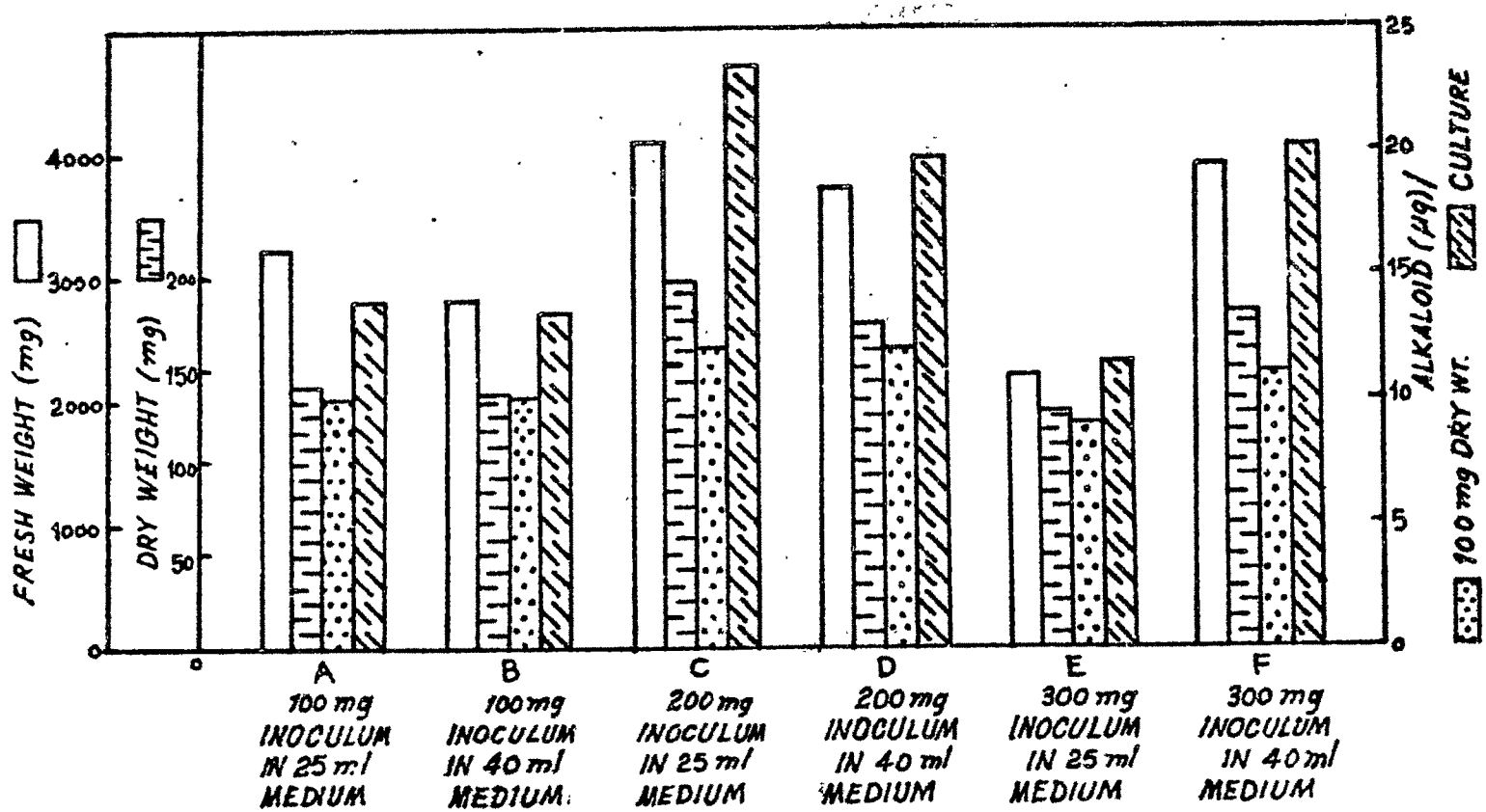

FIG 19. EFFECT OF INOCULUM SIZE/VOLUME OF MEDIUM ON GROWTH & ALKALOID PRODUCTION

SECTION - E

E - 1 : Effect of different sugars on growth and alkaloid production in cell suspensions.

Carbohydrates, mainly sugars, not only provide the energy source, but also supply carbon skeleton for the synthesis of secondary metabolites in plants. In present studies an experiment was carried out to find out the optimal source of energy and carbon for tissue growth and alkaloid production. The cultures were incubated on MS basal medium which lacked sugar for one week, before inoculation of the experiment to minimise any carry over effects. Measured aliquots of cell suspension (2 ml) weighing 200±20 mg by fresh weight were transferred to 25 ml of MS medium containing 2.0 mg/l 2,4-D and 0.4 mg/l kinetin in addition to 2% Fructose, 2% Glucose or 1% Glucose + 1% Fructose and 2% sucrose as control. The culture vessels were incubated at 25±2°C in continuous light for a period of 25 days and then harvested for the determination of growth and alkaloid production.

Both Fructose and Glucose were found to be inferior carbon sources compared to sucrose, both for growth of tissue as well as alkaloid production (Table 14, Fig. 20). Glucose was found to be a better source of carbon than fructose and glucose in combination with fructose was also observed to be a better source of carbon compared to fructose alone, as it

Table - 14. Effect of various sugars on growth and alkaloid production in E. alsinoides suspension cultures.

Inoculum : 200±20 mg tissue by fresh weight (dry weight 12.01 mg) in 25 ml of MS medium supplemented with -

(A) 2 mg/l 2,4-D + 0.4 mg/l K + 2% sucrose (CONTROL)

(B) 2 mg/l 2,4-D + 0.4 mg/l K + 2% glucose

(C) 2 mg/l 2,4-D + 0.4 mg/l K + 2% fructose

(D) 2 mg/l 2,4-D + 0.4 mg/l K + 1% glucose + 1% fructose.

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

Treatment	Fresh weight (mg/cult.)	Dry weight (mg/cult.)	Alkaloid (%)	Alkaloid (µg/cult.)
A	4006.24 (±13.265)	197.02 (±3.801)	0.012	23.6
B	3236.28 (±11.236)	171.91 (±2.145)	0.011	18.9
C	2232.28 (± 7.631)	134.01 (±1.251)	0.009	12.1
D	3002.36 (± 9.156)	168.01 (±1.701)	0.01	16.8

Data represents average of five replicates.

Figures in parenthesis represent standard error.

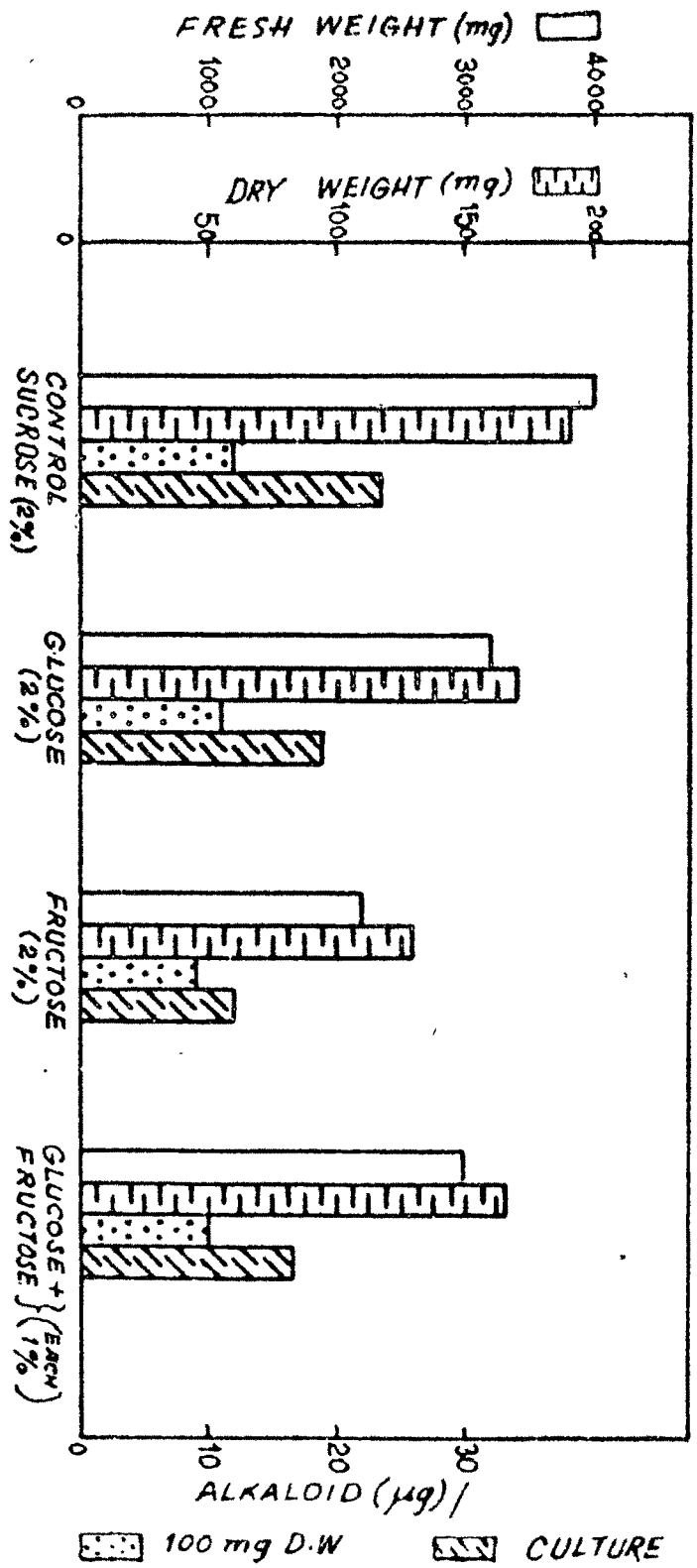

FIG. 20 - EFFECT OF VARIOUS SUGARS ON GROWTH & ALKALOID PRODUCTION

supported more growth of tissue and higher alkaloid production. Growth as well as alkaloid production was maximum in 2% sucrose medium.

E - 2 : Effect of sucrose levels on growth and alkaloid production and Tryptophan synthetase activity in suspension cultures.

To determine the optimal sucrose level for the synthesis of alkaloid and growth of tissue, MS medium was added with 0.0, 1.0, 2.0 and 4.0% sucrose in addition to 2.0 mg/l 2,4-D and 0.4 mg/l kinetin. Measured aliquots of cell suspension weighing 200±20 mg tissue by fresh weight were transferred to 25 ml of culture media. The culture vessels were incubated at 25±2°C in continuous light for a period of 30 days.

A fixed number of replicates was harvested at the interval of every 5 days for periodic measurement of tissue growth and alkaloid production. Of the different levels of sucrose tried, 2% sucrose was found to be more effective for growth as well as alkaloid production. The growth of tissue was comparatively negligible in medium containing no sucrose. Alkaloid production was also reduced compared to the cultures in 2% sucrose (Table 15, Fig. 21). Addition of 1% sucrose enhanced the growth of tissue as well as alkaloid production considerably (Table 16, Fig. 22); whereas 2% sucrose supported maximum growth of the tissue as well as alkaloid synthesis (Table 17, Fig. 24). Further increase

Table - 15. Periodic changes in growth, alkaloid production and tryptophan synthetase activity in E. alsinoides suspension cultures in absence of sucrose.

Inoculum : 200±20 mg tissue by fresh weight (dry weight 12.01 mg) in 25 ml of MS medium containing no sucrose but supplemented with 2.0 mg/l 2,4-D and 0.4 mg/l kinetin.

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

Days	Fresh weight (mg/cult.)	Dry weight (mg/cult.)	Alkaloid		Tryptophan synthetase	
			(%)	(µg/cult.)	(units/mg protein)	(units/cult.)
0	200.00 (± 1.517)	12.01 (±0.231)	0.009	-	-	-
5	225.00 (± 1.232)	12.12 (±0.872)	0.004	0.48	-	-
10	227.02 (± 1.861)	12.21 (±0.123)	0.004	0.49	-	-
15	250.17 (± 1.521)	13.01 (±0.131)	0.004	0.52	-	-
20	280.07 (± 1.421)	14.57 (±0.107)	0.004	0.58	-	-
25	292.16 (± 1.514)	14.87 (±0.103)	0.003	0.45	-	-
30	291.86 (± 1.436)	14.85 (±0.371)	0.003	0.45	-	-

Data represents average of five replicates.

Figures in parenthesis represent standard error.

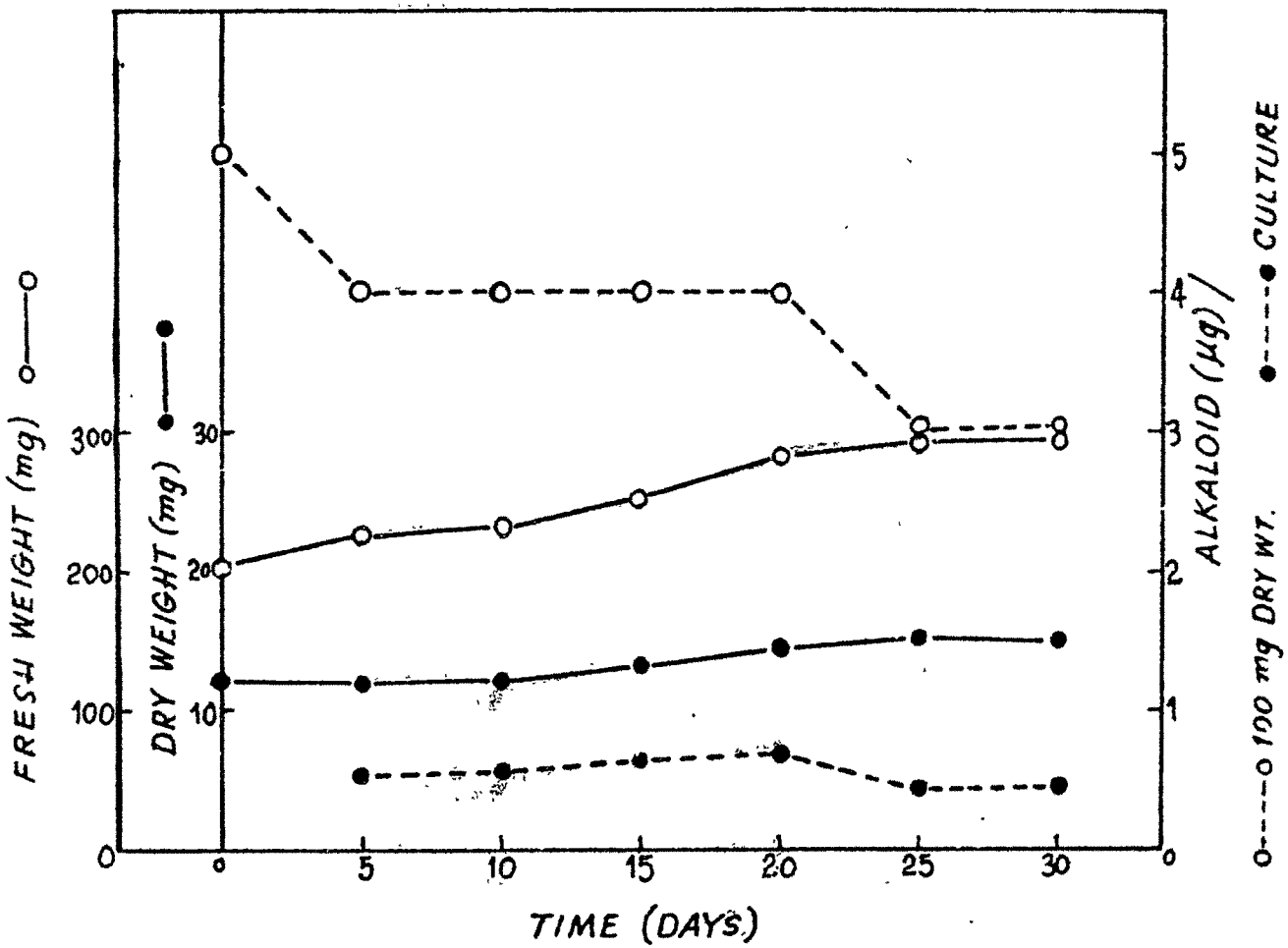

FIG. 21. EFFECT OF NIL SUCROSE ON GROWTH & ALKALOID PRODUCTION

Table - 16. Periodic changes in growth, alkaloid production and tryptophan synthetase activity in E. alsinoides suspension cultures at 1% sucrose level.

Inoculum : 200±20 mg tissue by fresh weight (dry weight 12.01 mg) in 25 ml of MS medium containing 1% sucrose and supplemented with 2.0 mg/l 2,4-D and 0.4 mg/l kinetin.

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

Days	Fresh weight (mg/cult.)	Dry weight (mg/cult.)	Alkaloid		Tryptophan synthetase	
			(%)	(µg/cult.)	(units/mg protein)	(units/cult.)
0	200.00 (±1.517)	12.01 (±0.231)	0.009	-	-	-
5	482.9 (±1.417)	28.8 (±0.148)	0.006	1.7	-	-
10	1019.8 (± 3.56)	83.3 (±0.178)	0.006	4.99	-	-
15	1416.2 (± 3.008)	100.1 (±1.015)	0.007	7.0	2.758	14.162
20	1554.6 (± 3.625)	101.2 (±1.113)	0.009	9.1	12.929	77.7
25	2533.6 (± 7.441)	123.0 (±1.171)	0.011	13.5	13.636	94.987
30	2535.6 (± 6.1232)	123.1 (±1.143)	0.01	12.3	-	-

Data represents average of five replicates.

Figures in parenthesis represent standard error.

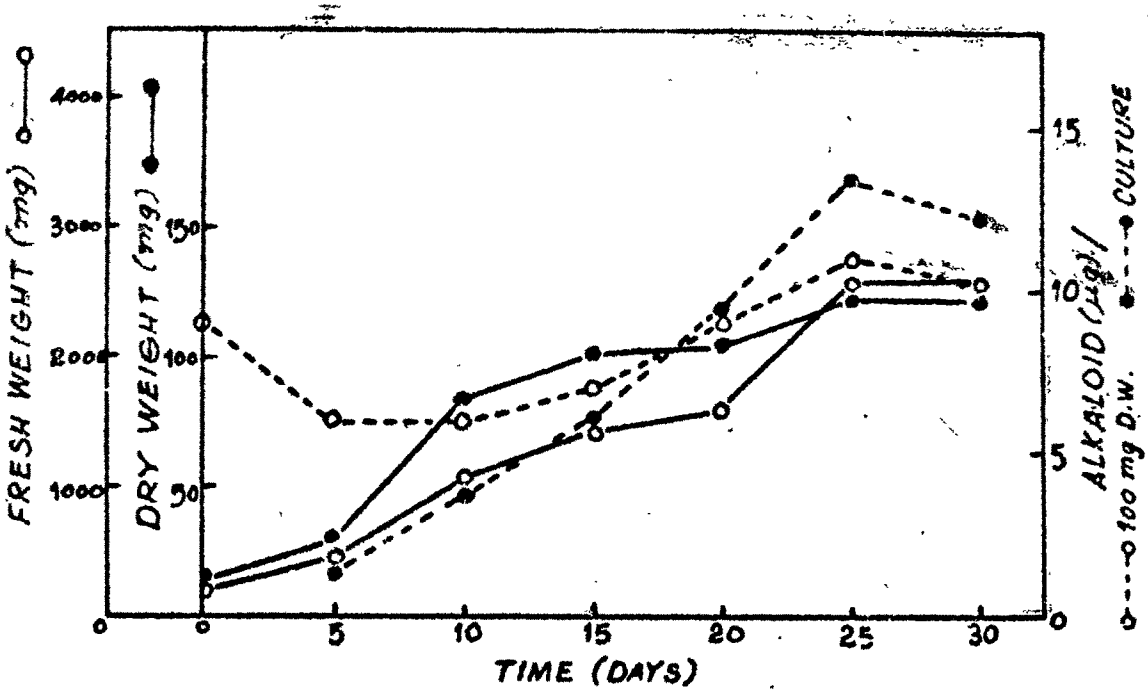

FIG. 22-EFFECT OF 1% SUCROSE ON GROWTH & ALKALOID PRODUCTION

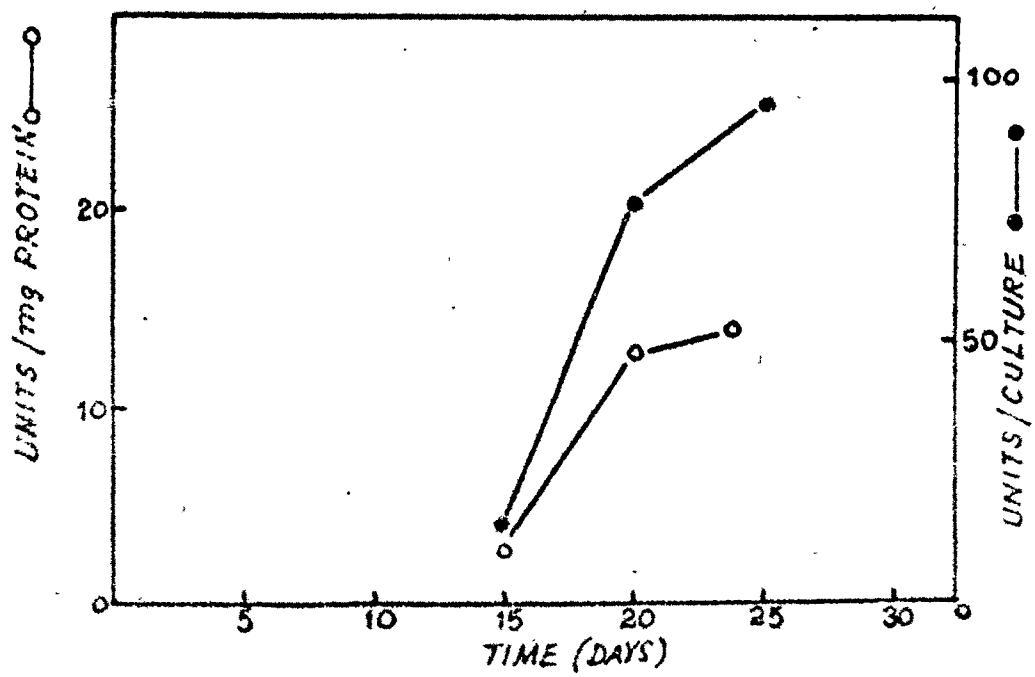

FIG. 23-EFFECT OF 1% SUCROSE ON TRYPTOPHAN SYNTHETASE ACTIVITY

Table - 17. Periodic changes in growth, alkaloid production and tryptophan synthetase activity in E. alsinoides suspension cultures at 2% sucrose level.

Inoculum : 200±20 mg tissue by fresh weight (dry weight 12.01 mg) in 25 ml of MS medium containing 2% sucrose and supplemented with 2.0 mg/l 2,4-D and 0.4 mg/l kinetin.

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

Days	Fresh weight (mg/cult.)	Dry weight (mg/cult.)	Alkaloid		Tryptophan synthetase	
			(%)	(µg/cult.)	(units/mg protein)	(units/cult.)
0	200.00 (±1.517)	12.01 (±0.231)	0.009	-	-	-
5	630.74 (±4.714)	40.94 (±1.131)	0.006	2.45	-	-
10	1100.56 (±2.398)	70.12 (±1.291)	0.006	4.2	20.228	64.48
15	1482.1 (±3.881)	98.14 (±1.968)	0.009	8.85	27.487	148.10
20	2481.2 (± 9.611)	141.1 (±1.321)	0.01	14.1	45.716	198.5
25.	4092.6 (±14.261)	198.8 (±3.123)	0.012	23.856	42.124	170.732
30	4088.6 (±29.186)	197.9 (±3.781)	0.01	19.8	-	-

Data represents average of five replicates.

Figures in parenthesis represent standard error.

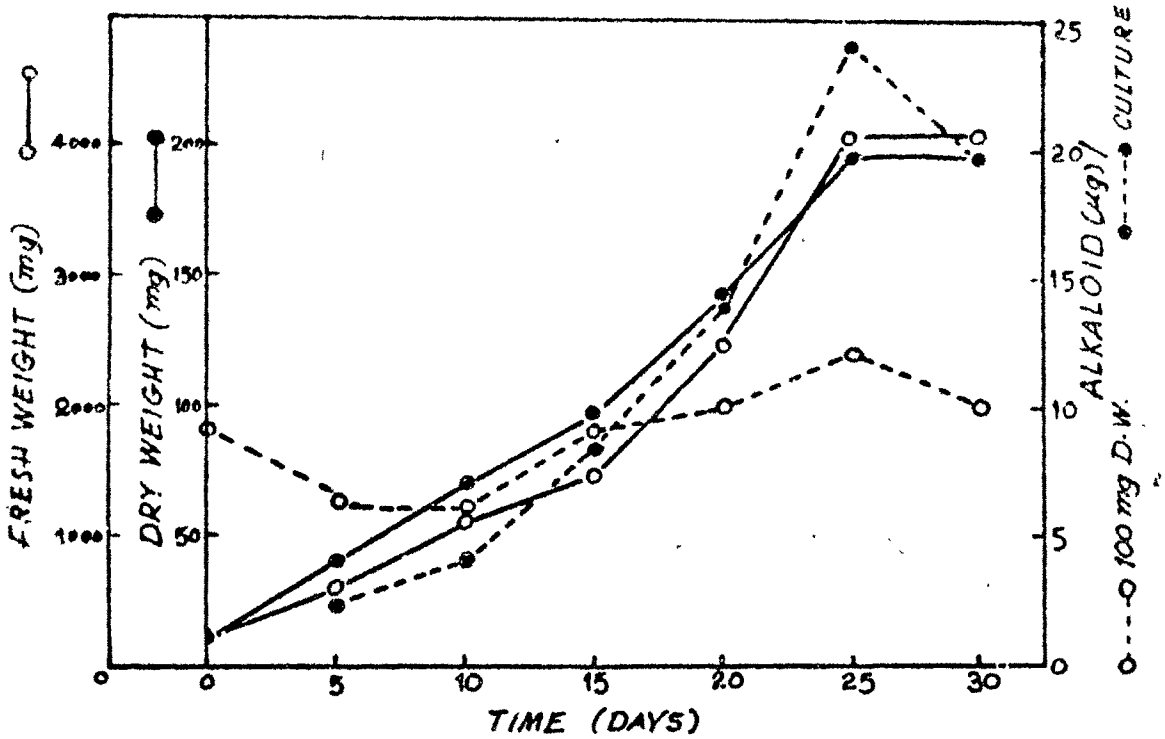

FIG.24-EFFECT OF 2% SUCROSE ON GROWTH & ALKALOID PRODUCTION

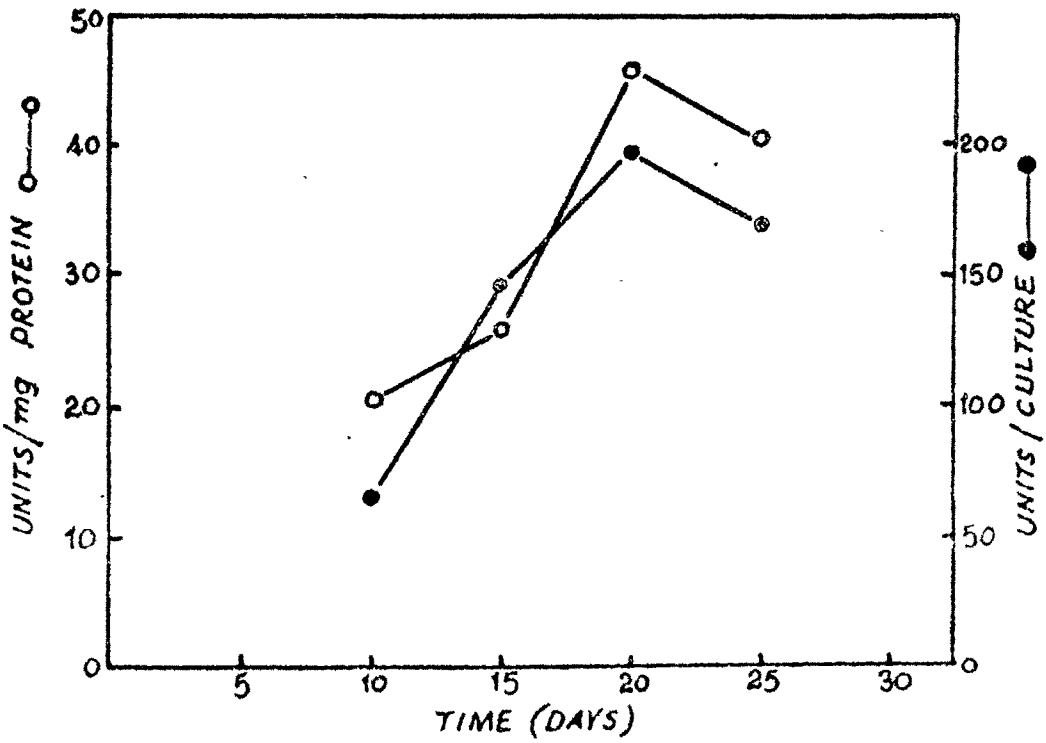

FIG.25-EFFECT OF 2% SUCROSE ON TRYPTOPHAN SYNTHETASE ACTIVITY

in sucrose level, however, reduced the alkaloid synthesis. Dry weight of the cultures, on the other hand, was appreciably higher at 4% sucrose compared to the control (Table 18, Fig. 26).

Enzyme assay was carried out at the intervals of 5 days till day 30 of culture. Specific activity of tryptophan synthetase per milligram protein in cultures containing 1% sucrose is presented in Table 16, Fig. 23. Tryptophan synthetase activity was maximum at 2% sucrose, whereas tissues growing in 1% sucrose had comparatively less activity. In case of 1% sucrose level enzyme activity was not detected until day 15, then it increased till day 25. Day 25 recorded peak activity and thereafter it declined, day 30 showing no activity. At the highest level of sucrose tested (4%), the tryptophan synthetase activity was detected from day 10 onwards, day 15 recording peak activity. The activity declined on day 20 and then it was not detected in the tissues (Table 18, Fig. 27). The enzyme activity was maximum at 2% sucrose level, whereas the lower and higher levels of sucrose showed comparatively less activity than in 2% sucrose (Table 17, Fig. 25). In absence of sucrose the enzyme activity was not detected.

Table - 18. Periodic changes in growth, alkaloid production and tryptophan synthetase activity in E. alsinoides suspension cultures at 4% sucrose level.

Inoculum : 200±20 mg tissue by fresh weight (dry weight 12.01 mg) in 25 ml of MS medium containing 4% sucrose and supplemented with 2.0 mg/l 2,4-D and 0.4 mg/l Kinetin.

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

Days	Fresh weight (mg/cult.)	Dry weight (mg/cult.)	Alkaloid		Tryptophan synthetase	
			(%)	(µg/cult.)	(units/mg protein)	(units/cult.)
0	200.00 (±1.517)	12.01 (±0.231)	0.009	-	-	-
5	502.1 (±1.186)	29.2 (±0.447)	0.006	1.8	-	-
10	1021.3 (±1.769)	84.1 (±0.116)	0.006	5.0	12.0	76.597
15	1781.5 (±3.186)	122.1 (±0.218)	0.006	7.3	13.33	133.575
20	2850.3 (±5.098)	214.5 (±1.383)	0.007	15.0	11.363	110.125
25	3446.2 (±6.164)	219.3 (±1.392)	0.009	19.7	-	-
30	4021.3 (±6.813)	230.3 (±2.412)	0.009	21.0	-	-

Data represents average of five replicates.

Figures in parenthesis represent standard error.

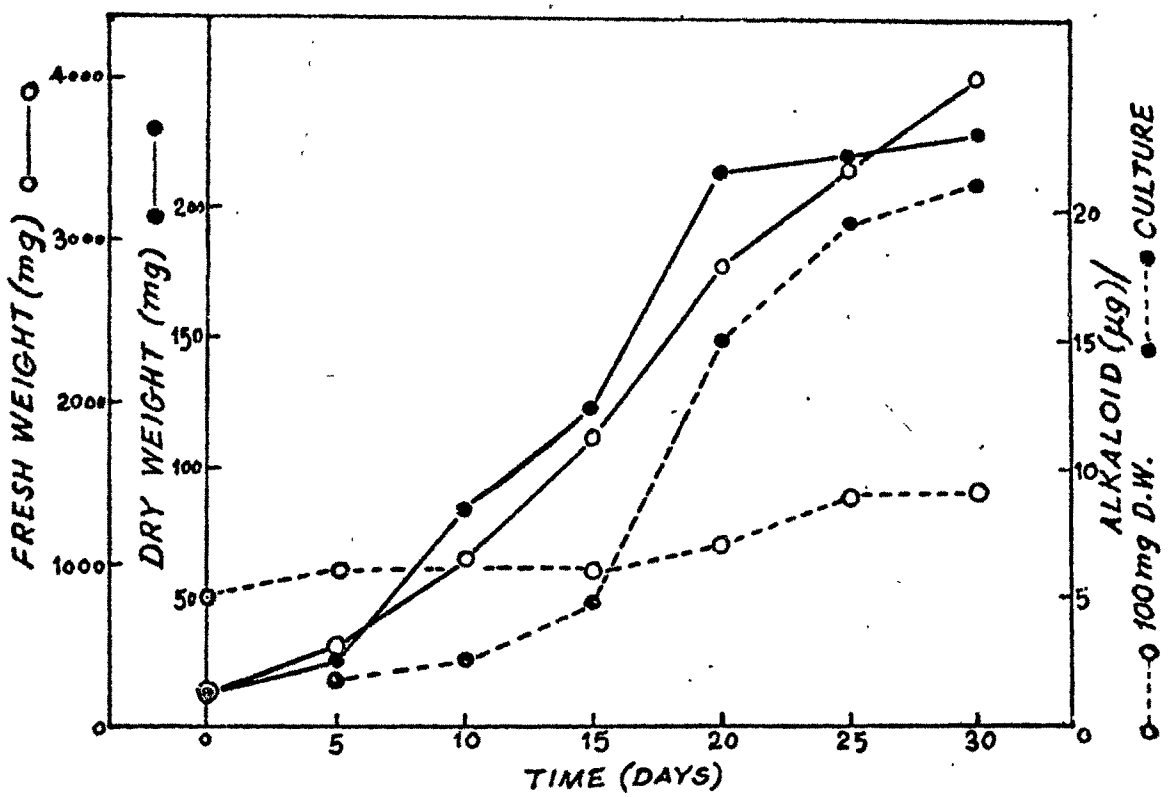

FIG. 26 - EFFECT OF 4% SUCROSE ON GROWTH & ALKALOID PRODUCTION

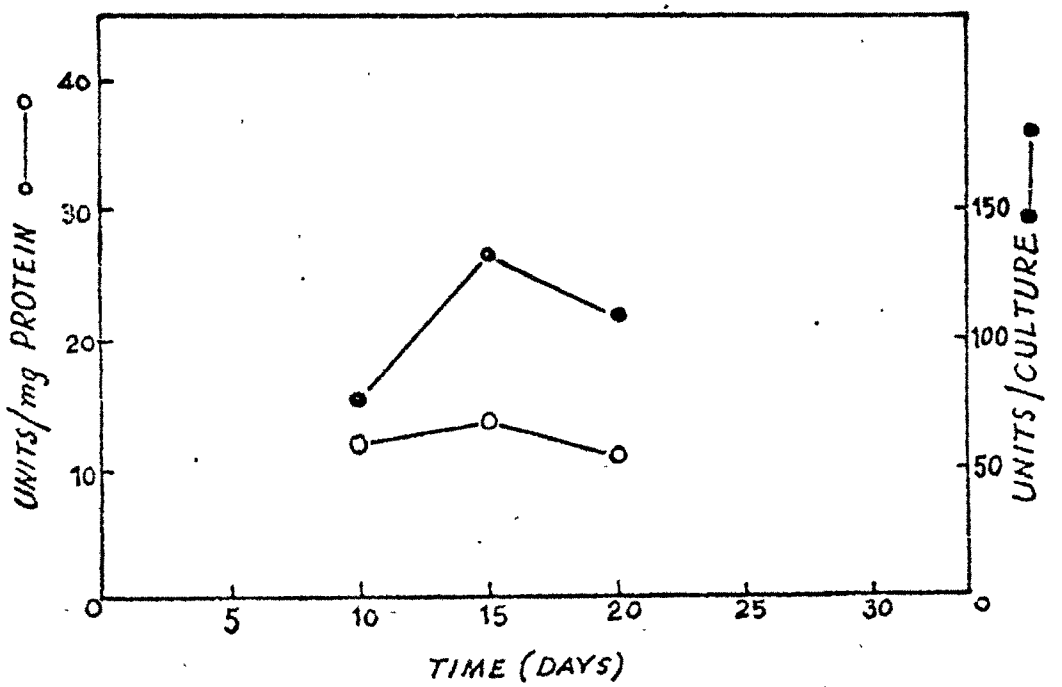

FIG 27 - EFFECT OF 4% SUCROSE ON TRYPTOPHAN SYNTHETASE ACTIVITY

SECTION - F

Effect of Total Nitrogen level on growth and alkaloid production in suspension cultures.

Different sources of nitrogen as well as different levels of nitrogen are known to influence growth of tissues and production of secondary metabolites. In the present studies, the effect of total nitrogen in the form of NH_4NO_3 and KNO_3 has been examined. MS/basal medium contained 840 mg/l nitrogen as ammonium nitrate and potassium nitrate. This nitrogen level has been altered to find out the effect of its different levels on growth of tissue and alkaloid production. As in previous cases, the culture was incubated for one week in nitrogen free MS medium (with all hormonal supplements and sucrose) to minimise the carry over effects. Measured aliquots of cell suspension weighing 200±20 mg by fresh weight were transferred to 25 ml of MS medium (supplemented with 2.0 mg/l 2,4-D and 0.4 mg/l kinetin in addition to 2% sucrose) containing different nitrogen levels. The different levels of nitrogen tested were Nitrogen free medium, 840 mg/l (control), 1680 mg/l and 3360 mg/l. The culture vessels were incubated at 25±2°C in continuous light for a period of 25 days.

The culture vessels were harvested after 25 days of incubation for the measurement of growth of tissue and alkaloid production. The results are presented in Table 19 and Fig. 28.

In cultures where nitrogen was absent, the fresh weight increase was about 5 fold and dry weight increase was 4 fold; whereas in the control the respective increases were 20 fold and 18 fold. In cultures containing double the amount of nitrogen than control, a fresh weight increase of 8 fold and dry weight increase of 9 fold were registered. At the highest level of nitrogen tested (3360 mg/l), the fresh weight increase was only 4 fold; while the dry weight increase was 5 fold; i.e. growth was as low at 4 fold N level as when it was totally absent.

Alkaloid production was also maximum at the standard levels of Nitrogen (840 mg/l). In absence of nitrogen, there was complete inhibition of alkaloid production besides the degradation of initially present alkaloids. Increased levels of nitrogen than in control also showed a suppressive effect on alkaloid production. The results presented in Table 19 and Fig. 28, thus clearly show that the normal supply of nitrogen (840 mg/l) as in MS standard medium supported maximum growth of tissue and alkaloid production.

Table - 19. Effect of different Nitrogen levels on growth and alkaloid production in E. alsinoides suspension cultures.

Inoculum : 200±20 mg tissue by fresh weight (dry weight 11.08 mg) in 25 ml of MS medium supplemented with -

(A) 2.0 mg/l 2,4-D + 0.4 mg/l K + 840 mg/l Nitrogen (Control) + 2% Sucrose.

(B) 2.0 mg/l 2,4-D + 0.4 mg/l K + zero Nitrogen + 2% sucrose.

(C) 2.0 mg/l 2,4-D + 0.4 mg/l K + 1680 mg/l Nitrogen + 2% sucrose.

(D) 2.0 mg/l 2,4-D + 0.4 mg/l K + 3360 mg/l Nitrogen + 2% sucrose.

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

Medium	Fresh weight (mg/cult.)	Dry weight (mg/cult.)	Alkaloid (%)	Alkaloid (µg/cult.)
A	4006.24 (±13.265)	197.62 (±3.801)	0.012	23.6
B	1021.8 (± 5.671)	67.23 (±1.932)	0.003	2.02
C	1823.07 (± 7.212)	99.01 (±1.521)	0.008	7.9
D	824.73 (± 1.871)	52.01 (±1.212)	0.005	2.6

Data represents average of five replicates.

Figures in parenthesis represent standard error.

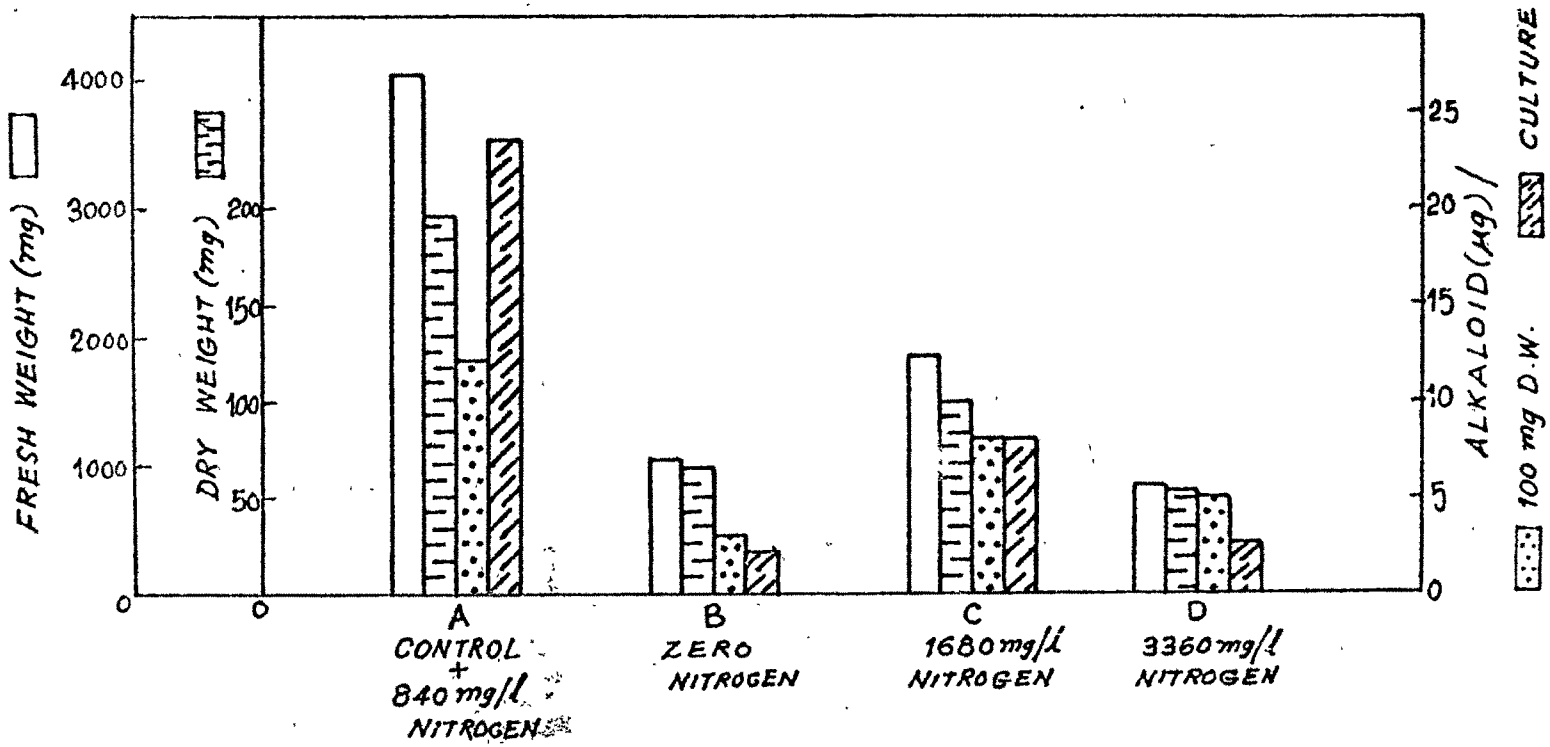

FIG. 28-EFFECT OF DIFFERENT NITROGEN LEVELS ON GRROWTH & ALKALOID PRODUCTION

SECTION - G

Effect of different levels of Microelement and Macroelement salts on growth and alkaloid production

The combinations of macroelement and microelement salts studied were :

Microelement salt \ Macroelement salt	0	X 1	X 2
0			
X 1		Control	
X 2			

The results of the experiment are present in Table 20 and Figs. 29, 30, 31. Of the various combinations of microelements and macroelements studied, 1 microelement + 2 macroelement, as well as 2 microelement + 2 macroelement induced maximum growth of tissue and alkaloid production (Fig. 31). Absence of microelements and macroelements in the medium had adverse effect on growth and alkaloid production (Fig. 29). In the absence of microelements and macroelements, the tissue became dry and brownish in colour with practically no growth. The alkaloid production was also completely suppressed, recording a very low percentage of alkaloid on day 25.

Incorporation of macroelements, at increasing levels, without microelements in the medium did support some growth and alkaloid production, but it was less than the control. Microelements alone, at various levels, in the absence of macroelements could not promote growth or alkaloid production. The maximum growth of tissue was noticed at the highest levels of microelements and macroelements (2 + 2), where the fresh weight increase was about 19 fold and dry weight increase was about 14 fold; while control showed a fresh weight increase of 17 fold and dry weight increase of 13 fold. On the other hand, maximum alkaloid production was registered at 1 micro + 2 macro and also at 2 micro + 2 macroelement salts levels.

Table - 20. Effect of different levels of Macroelements and Microelements on growth and alkaloid production in *E. alsinoides* suspension cultures.

Inoculum : 200±20 mg tissue by fresh weight (dry weight 11.08 mg) in 25 ml of MS medium with various combinations of Microelements and Macroelements containing 2% sucrose and supplemented with 2.0 mg/l 2,4-D and 0.4 mg/l K.

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

Treatment		Fresh weight (mg/cult.)	Dry weight (mg/cult.)	Alkaloid	
Micro-element	Macro-element			(%)	(µg/cult.)
Control (1 + 1)		3450.02 (± 9.632)	149.01 (±2.123)	0.01	14.9
	0	261.24 (±1.432)	11.47 (±0.123)	0.004	0.45
0 +	1	2480.2 (± 5.625)	116.12 (±2.861)	0.009	10.4
	2	2489.2 (± 4.065)	116.73 (±1.732)	0.015	17.5
	0	221.35 (± 2.212)	11.76 (±0.615)	0.005	0.6
1 +	1	3450.02 (± 9.632)	149.01 (±2.123)	0.01	14.9
	2	3675.24 (±10.0515)	152.2 (±3.161)	0.016	24.0
	0.	260.14 (±3.432)	11.23 (±0.326)	0.005	0.6
2 +	1	3491.12 (±11.632)	150.62 (±3.581)	0.013	19.6
	2	3781.2 (± 9.364)	158.02 (±3.124)	0.016	25.0

Data represents average of five replicates.

Figures in parenthesis represent standard error.

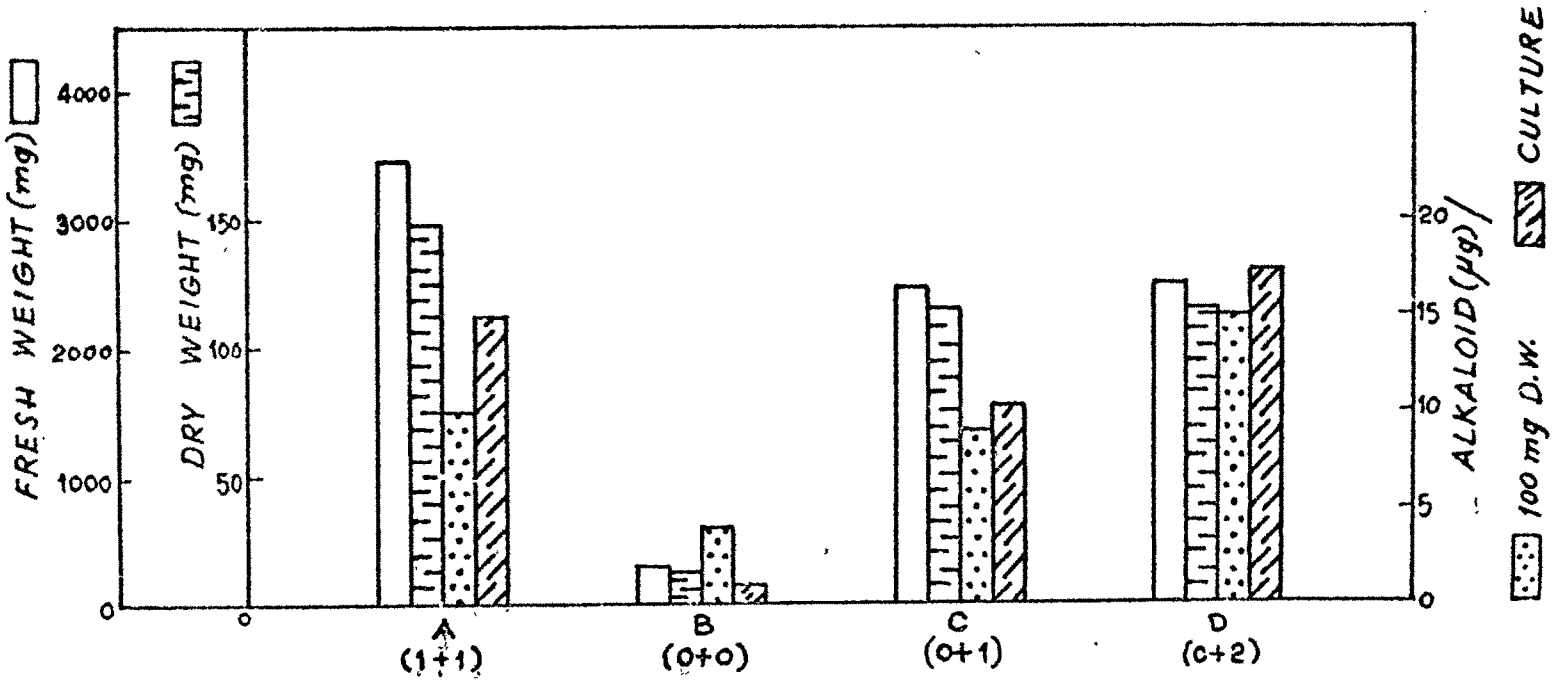

FIG.29-EFFECT OF DIFFERENT LEVELS OF MICRO & MACRO ELEMENTS ON GROWTH AND ALKALOID PRODUCTION

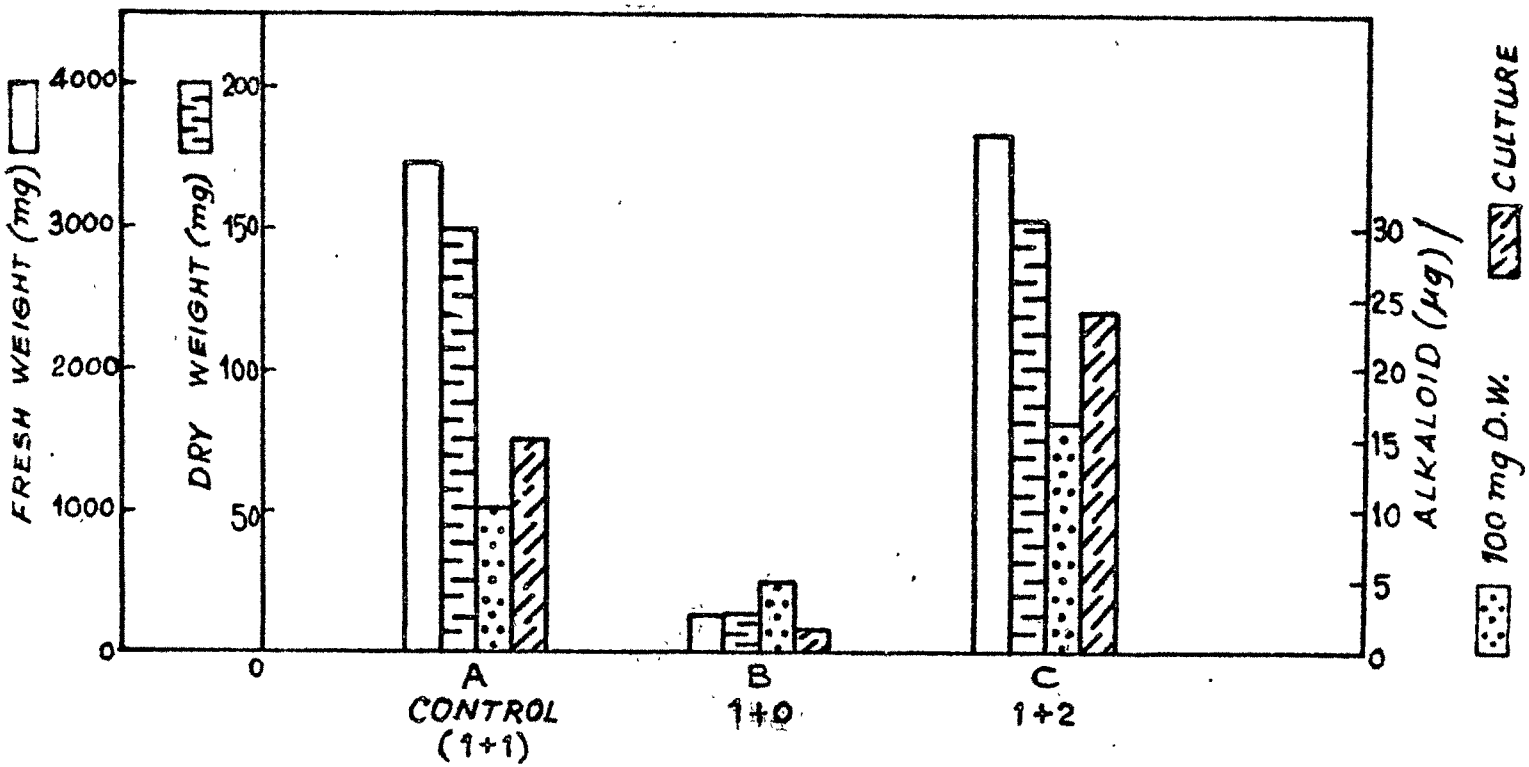

FIG. 30 - EFFECT OF DIFFERENT LEVELS OF MICRO & MACRO ELEMENTS ON GROWTH AND ALKALOID PRODUCTION.

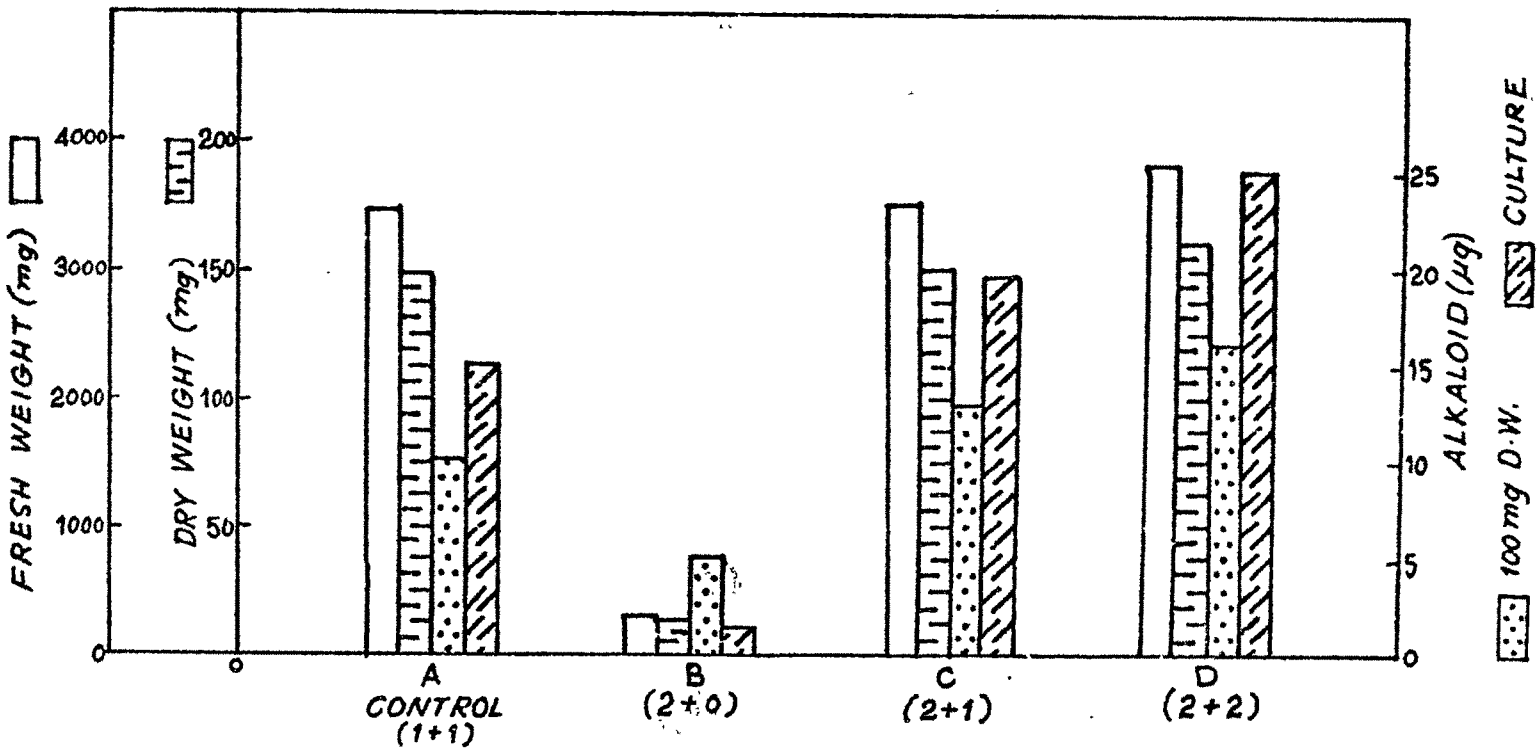

FIG. 31- EFFECT OF DIFFERENT LEVELS OF MICRO & MACRO ELEMENTS ON GROWTH AND ALKALOID PRODUCTION

SECTION - H

Effect of Tweens on growth and alkaloid production in Evolvulus suspension cultures.

Tweens are surface reactants which help in the uptake of different components from the medium. Hence the effect of Tweens were studied on growth of tissue as well as production of alkaloids. The different Tweens studied were Tween-40 (Palmitic acid), Tween-60 (Stearic acid) and Tween-80 (Oleic acid). In each case the following concentrations were tried.

Tween-40 : 0.2, 0.5, 1.0, 2.0% (v/v)
Tween-60 : 0.2, 0.5, 1.0, 2.0% (v/v)
Tween-80 : 0.2, 0.5, 1.0, 2.0% (v/v).

Measured aliquots of cell suspension weighing 200±20 mg by fresh weight were transferred to 25 ml of MS medium (supplemented with 2.0 mg/l 2,4-D and 0.4 mg/l kinetin in addition to 2% sucrose) containing 0.2, 0.5, 1.0 or 2.0% (v/v) of Tween 40, 60 or 80. The culture vessels were incubated at 25±2°C in continuous light for 25 days.

The culture vessels were harvested after 25 days of incubation for the determination of growth and alkaloid production. The growth of tissue was relatively less in all the Tween levels studied in comparison to the control. In the treatment with Tween-40, 0.2% (v/v) promoted maximum

growth of tissue compared to the other concentrations studied. Alkaloid production was however, more in 0.5% (v/v) compared to other concentrations (Table 21, Fig. 32). Growth was higher in the control, but at all Tween-40 levels except 2.0%, the alkaloid content was more than in the control.

In Tween-60 (Table 22, Fig. 33) and Tween-80 (Table 23, Fig. 34) both, a similar pattern in growth and alkaloid production was observed.

Among the three Tweens studied, Tween-80 was found to be more effective for production of alkaloid, whereas growth of tissue was more in Tween-40. Hence, for further experimental studies Tween-80 was used. Among the different concentrations of Tween-80 studied, 0.5% was found to be more effective in inducing alkaloid production. Tween-80 was therefore, added at 0.5% (v/v) concentration in the subsequent experiment.

In the following experiment Tween-80 (0.5%) was added periodically to study its effect on alkaloid production. It was added on day 0, day 5, day 10, day 15 and day 20 of culture. In all cases, the cultures were incubated for 25 days at 25±2°C in continuous light and the culture vessels were harvested on 25th day for measurement of growth and alkaloid production.

The results (Table 24, Fig. 35) indicated that addition of Tween-80 on day 0, was more effective for alkaloid production, white the growth of tissue was more in treatments where Tween was added on day 20.

Table - 21. Effect of Tween-40 on growth and alkaloid production in E. alsinoides suspension cultures.

Inoculum : 200±20 mg tissue by fresh weight (dry weight 11.08 mg) in 25 ml of MS medium containing 2% sucrose and supplemented with 2.0 mg/l 2,4-D and 0.4 mg/l kinetin and Tween-40.

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

Treatment Tween-40 (v/v)	Fresh weight (mg/cult.)	Dry weight (mg/cult.)	Alkaloid (%)	Alkaloid (µg/cult.)
Zero (Control)	3500.01 (±11.021)	150.73 (±3.0013)	0.01	15.1
0.2	2872.21 (±12.123)	131.25 (±2.816)	0.013	17.1
0.5	2012.34 (±13.013)	75.321 (±4.161)	0.016	12.0
1.0	2001.2 (± 7.841)	74.23 (±1.864)	0.012	8.9
2.0	1412.12 (± 7.124)	58.24 (±2.154)	0.008	4.7

Data represents average of five replicates.

Figures in parenthesis represent standard error.

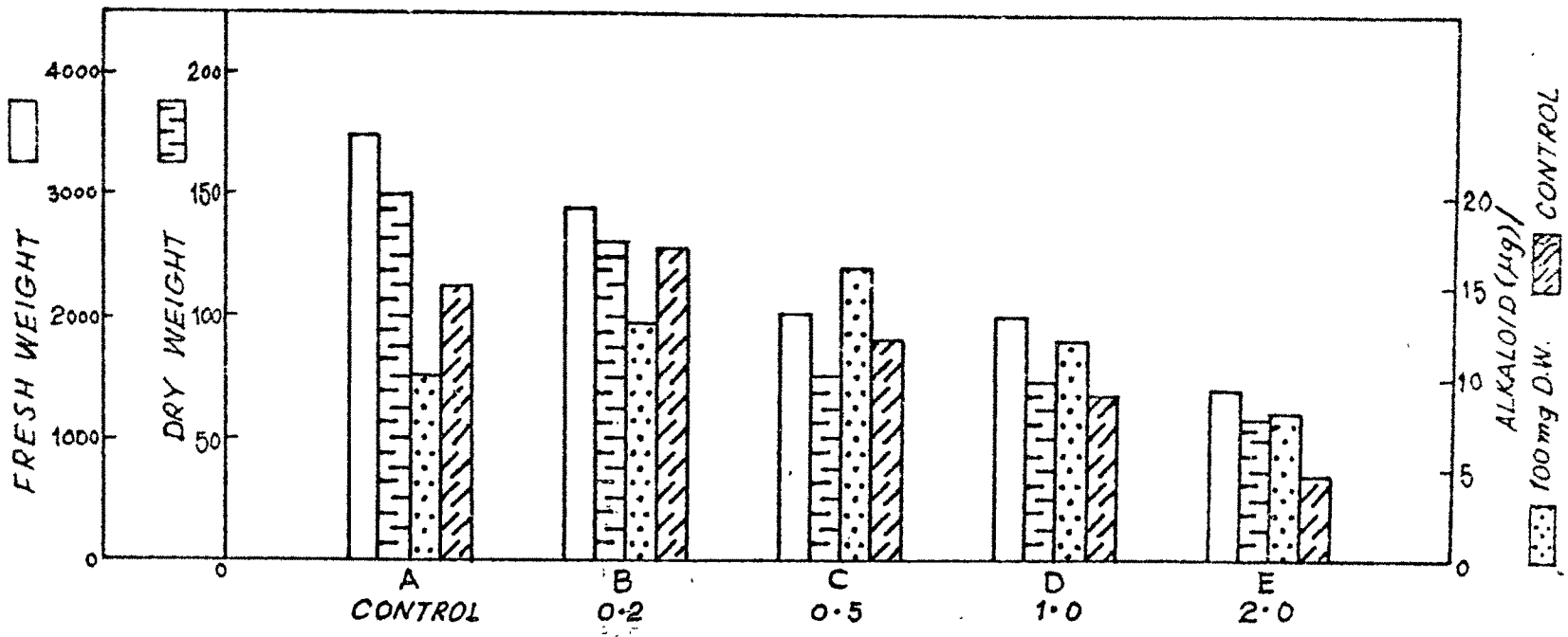

FIG. 32:- EFFECT OF TWEEN-40 ON GROWTH AND ALKALOID PRODUCTION

Table - 22. Effect of Tween-60 on growth and alkaloid production in E. alsinoides suspension cultures.

Inoculum : 200±20 mg tissue by fresh weight (dry weight 11.08 mg) in 25 ml of MS medium containing 2% sucrose and supplemented with 2.0 mg/l 2,4-D and Tween-60.

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

Treatment Tween-60 (v/v)	Fresh weight (mg/cult.)	Dry weight (mg/cult.)	Alkaloid (%)	Alkaloid (µg/cult.)
Zero (Control)	3500.01 (±11.021)	150.73 (±3.001)	0.01	15.1
0.2	2432.1 (±15.613)	127.25 (±7.865)	0.012	15.0
0.5	1912.013 (±13.541)	68.21 (±5.261)	0.015	10.2
1.0	1801.1 (±11.161)	62.21 (±3.184)	0.013	8.1
2.0	1523.2 (±12.061)	58.23 (±5.123)	0.009	5.0

Data represents average of five replicates.

Figures in parenthesis represent standard error.

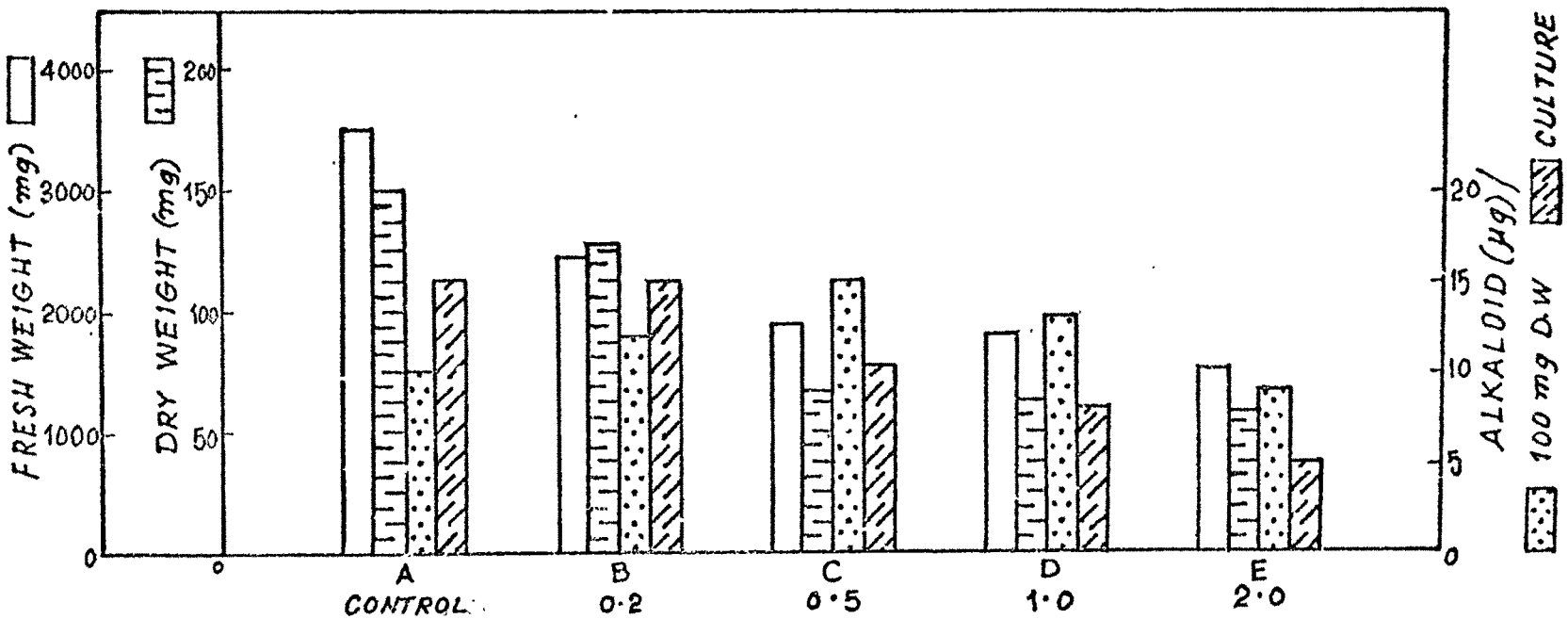

FIG.33-EFFECT OF TWEEN-60 ON GROWTH AND ALKALOID PRODUCTION

Table - 23. Effect of Tween-80 on growth and alkaloid production in E. alsinoides suspension cultures.

Inoculum : 200±20 mg tissue by fresh weight (dry weight 11.08 mg) in 25 ml of MS medium containing 2% sucrose and supplemented with 2.0 mg/l 2,4-D and 0.4 mg/l kinetin and Tween-80.

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

Treatment Tween-80 (v/v)	Fresh weight (mg/cult.)	Dry weight (mg/cult.)	Alkaloid (%)	Alkaloid (µg/cult.)
Zero (Control)	3500.01 (± 11.021)	150.73 (±3.001)	0.01	15.1
0.2	2221.2 (±13.001)	80.01 (±7.165)	0.013	10.4
0.5	1862.34 (± 9.642)	67.02 (±3.832)	0.017	11.3
1.0	1501.1 (±11.236)	63.01 (±1.678)	0.014	8.8
2.0	1012.1 (± 5.212)	58.01 (±1.378)	0.01	5.8

Data represents average of five replicates.

Figures in parenthesis represent standard error.

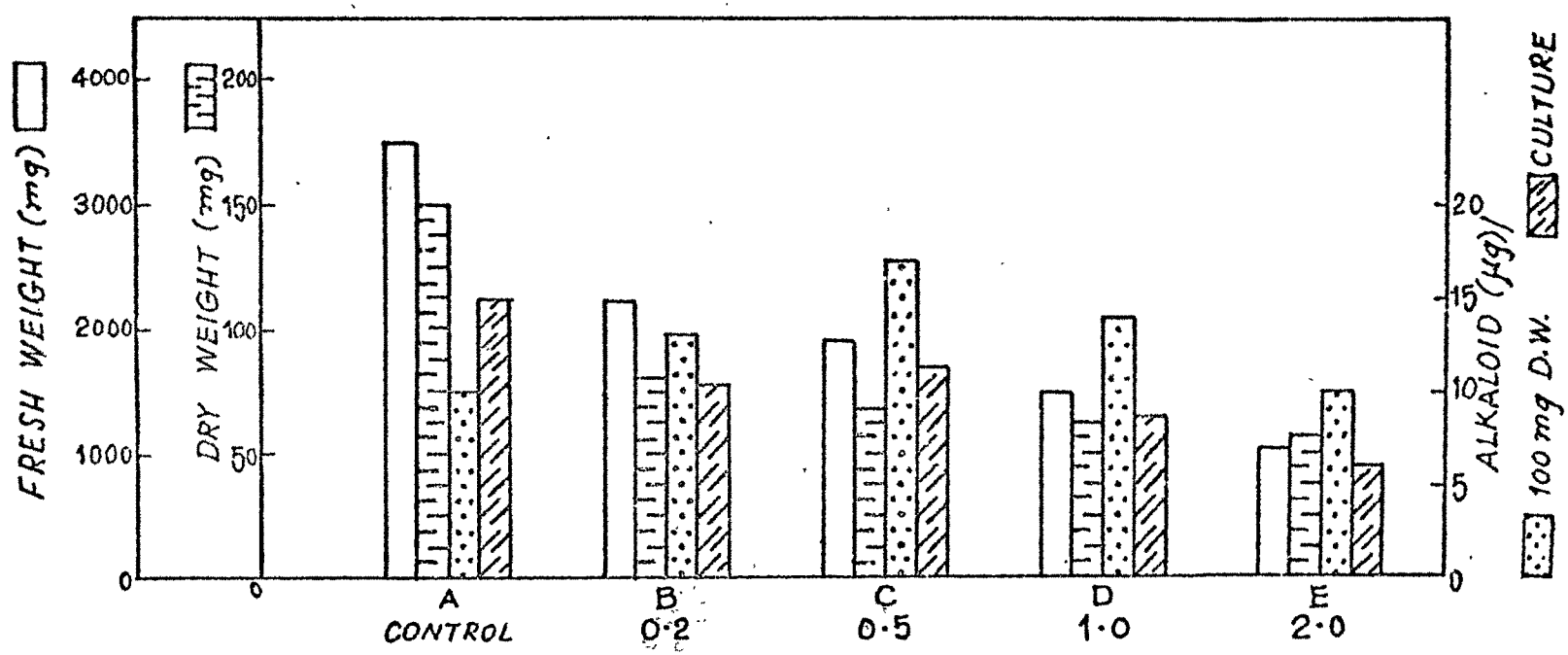

FIG. 34 – EFFECT OF TWEEN-80 ON GROWTH AND ALKALOID PRODUCTION

Table - 24. Effect of sequential (periodic) addition of Tween-80 on growth and alkaloid production in *E. alsinoides* suspension culture.

Inoculum : 200±20 mg tissue by fresh weight (dry weight 11.08 mg) in 25 ml of MS medium containing 2% sucrose and supplemented with 2.0 mg/l 2,4-D and 0.4 mg/l kinetin and 0.5 v/v Tween-80.

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

Tween-80 (0.5 v/v) Time of addition	Fresh weight (mg/cult.)	Dry weight (mg/cult.)	Alkaloid (%)	Alkaloid (µg/cult.)
Zero Tween (Control)	3501.21 (±10.021)	150.71 (±3.001)	0.01	15.1
5th day	1802.02 (± 7.123)	66.01 (±1.546)	0.016	10.2
10th day	1702.32 (± 5.832)	65.032 (±1.345)	0.15	9.75
15th day	1632.3 (± 3.872)	64.32 (±1.643)	0.013	8.4
20th day	2315.2 (± 9.165)	132.01 (±1.832)	0.013	17.0

Data represents average of five replicates.

Figures in parenthesis represent standard error.

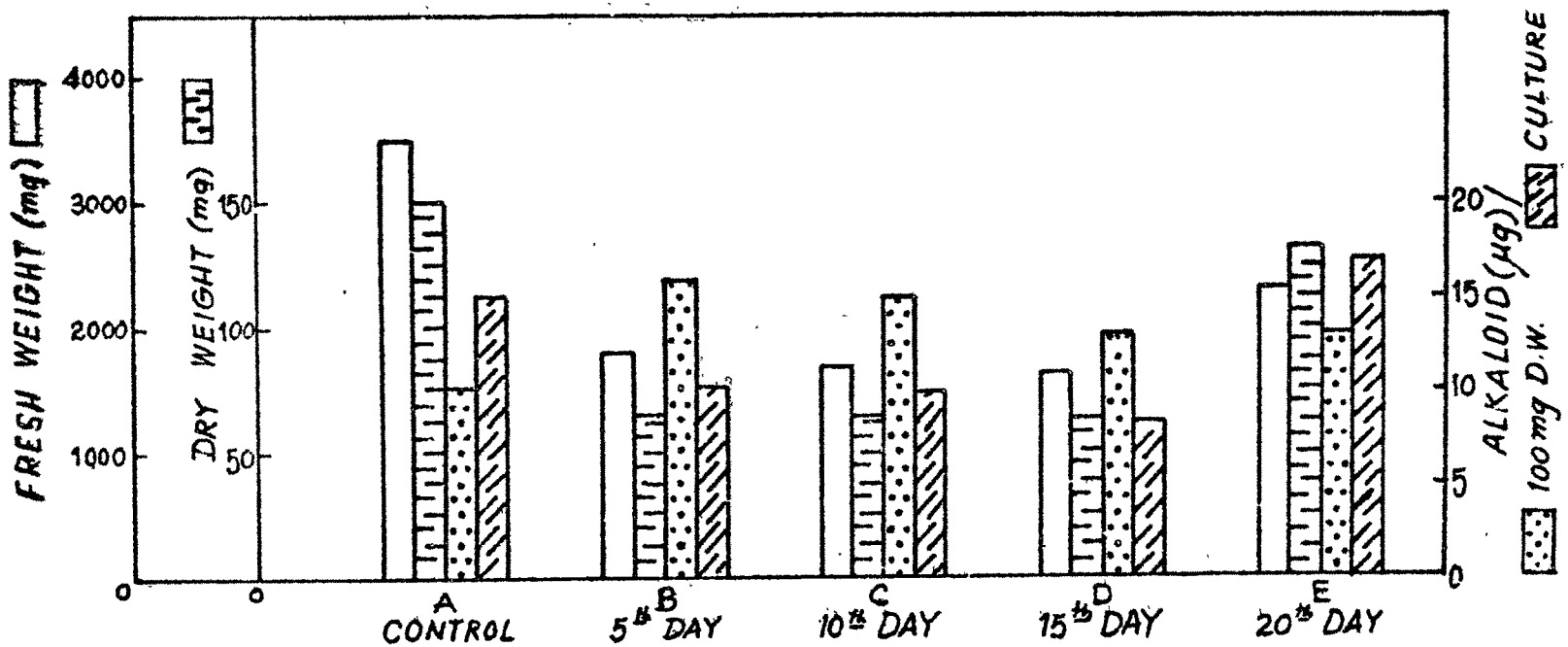

FIG. 35- EFFECT OF SEQUENTIAL ADDITION OF TWEEN-80 ON GROWTH AND ALKALOID PRODUCTION

SECTION - I

Effect of precursors on growth and alkaloid production in Evolvulus suspension cultures.

The main precursors of ergot alkaloids are Tryptophan, Mevalonic acid and Methionine. Voluminous work has been done on the effect of L-tryptophan on alkaloid production in Claviceps species and further the effect of early and late addition of Tryptophan to cultures has also been studied. Mevalonic acid supplies an Isoprene unit of ergot alkaloids. Methionine supplies the methyl group and Tryptophan forms the main skeleton of ergot alkaloids. Moreover, Anthranilic acid, Indole and Serine are the indirect precursors of ergot alkaloids as Tryptophan, the main precursor is produced from them. Anthranilic acid is converted to Indole, whereas Indole and Serine reacts together in the presence of Tryptophan synthetase to form Tryptophan. In addition to these precursors, various homologues of Tryptophan like Thiotryptophan, 5-methyl tryptophan etc. were also found to have stimulatory effect on alkaloid production. Keeping in view the above work, various experiments were carried out here with a higher plant system, the results obtained are described in the following subsections.

I - 1 : Effect of L-tryptophan on growth, alkaloid production and Tryptophan synthetase activity.

The different concentrations of Tryptophan incorporated in

the medium were 2 mM, 5 mM and 10 mM. Measured aliquots of cell suspension weighing 200±20 mg by fresh weight were transferred to 25 ml of MS medium (supplemented with 2.0 mg/l 2,4-D and 0.4 mg/l kinetin in addition to 2% sucrose) containing 2, 5 or 10 mM L-tryptophan. The culture vessels were incubated at 25±2°C in continuous light for 30 days. A fixed number of replicates was harvested at the intervals of 5 days for the measurement of growth, alkaloid production and enzyme assay. The results are presented in Tables 25-27 and Figs. 36-41.

Addition of 2 mM L-tryptophan had a promotory effect on alkaloid production, but growth of tissue was reduced in comparison to normal (control) cultures (Table 25, Fig. 36). Increasing concentrations of L-tryptophan had profound effect on alkaloid production, 10 mM Tryptophan recording maximum production of alkaloids (Table 27, Fig. 40). However, the growth of tissue was inhibited in cultures containing 10 mM Tryptophan, recording only 6 fold increase in fresh weight and dry weight during the course of culture. At 5 mM Tryptophan level also, there was comparatively more alkaloid production, but reduced growth of tissue (Table 26, Fig. 38), recording 7 fold increase in fresh weight and 6 fold increase in dry weight. The tissue turned dry and brownish in colour at all levels of Tryptophan tested.

Tryptophan synthetase activity was not recorded till day 10 in all the levels of Tryptophan examined. In case of 2 mM

Table - 25. Periodic changes in growth, alkaloid production and tryptophan synthetase activity in E. alsinoides suspension cultures with L-tryptophan levels.

Inoculum : 200±20 mg tissue by fresh weight (dry weight 11.08 mg) in 25 ml of MS medium containing 2% sucrose and supplemented with 2.0 mg/l 2,4-D and 0.4 mg/l Kinetin and 2 mM L-tryptophan.

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

Days	Fresh weight (mg/cult.)	Dry weight (mg/cult.)	Alkaloid		Tryptophan synthetase	
			(%)	(µg/cult.)	(units/mg protein)	(units/cult.)
0	200.0 (± 1.232)	11.08 (±0.125)	0.008	-	-	-
5	321.9 (± 1.561)	15.6 (±0.391)	0.006	0.94	-	-
10	934.4 (± 2.036)	46.8 (±0.596)	0.008	3.7	13.2	53.34
15	1066.8 (± 2.498)	57.8 (±0.527)	0.008	4.6	32.83	160.497
20	1524.4 (± 4.157)	67.1 (±1.217)	0.012	8.1	25.0	150.44
25	2701.2 (± 5.177)	110.1 (±1.908)	0.014	15.4	21.87	145.855
30	2713.6 (±11.301)	110.9 (±1.182)	0.012	13.3	-	-

Data represents average of five replicates.

Figures in parenthesis represent standard error.

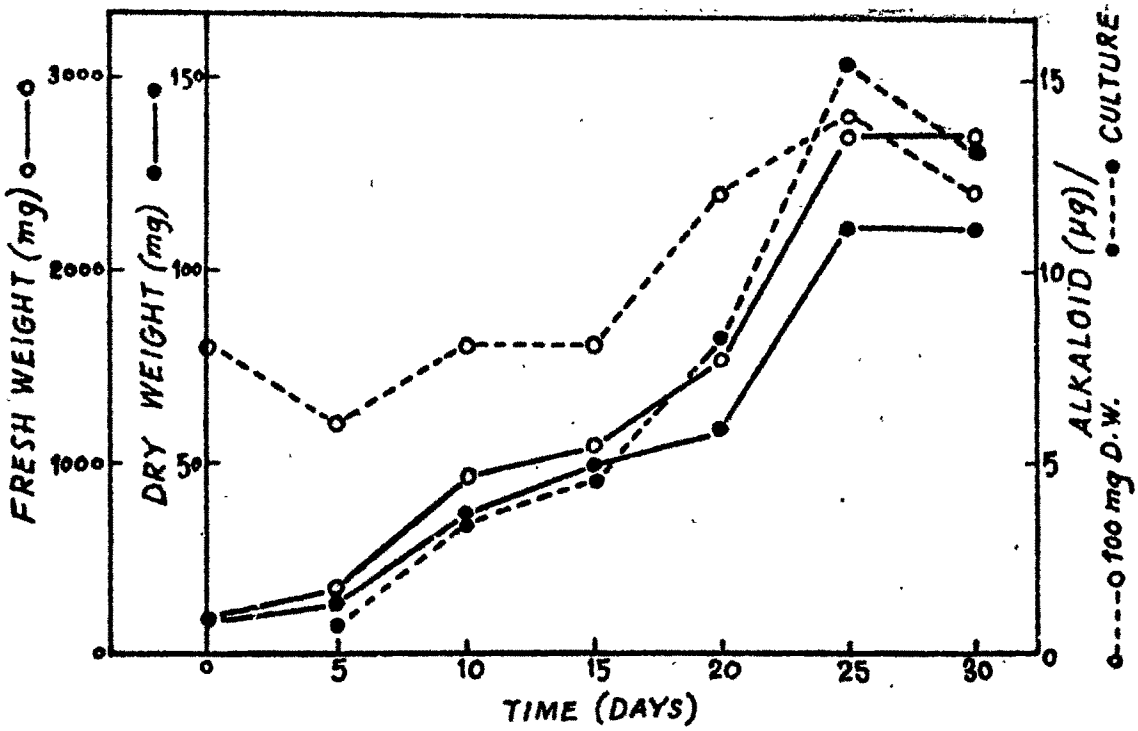

FIG. 36 - EFFECT OF 2 mM, L-TRYPTOPHAN ON GROWTH & ALKALOID PRODUCTION

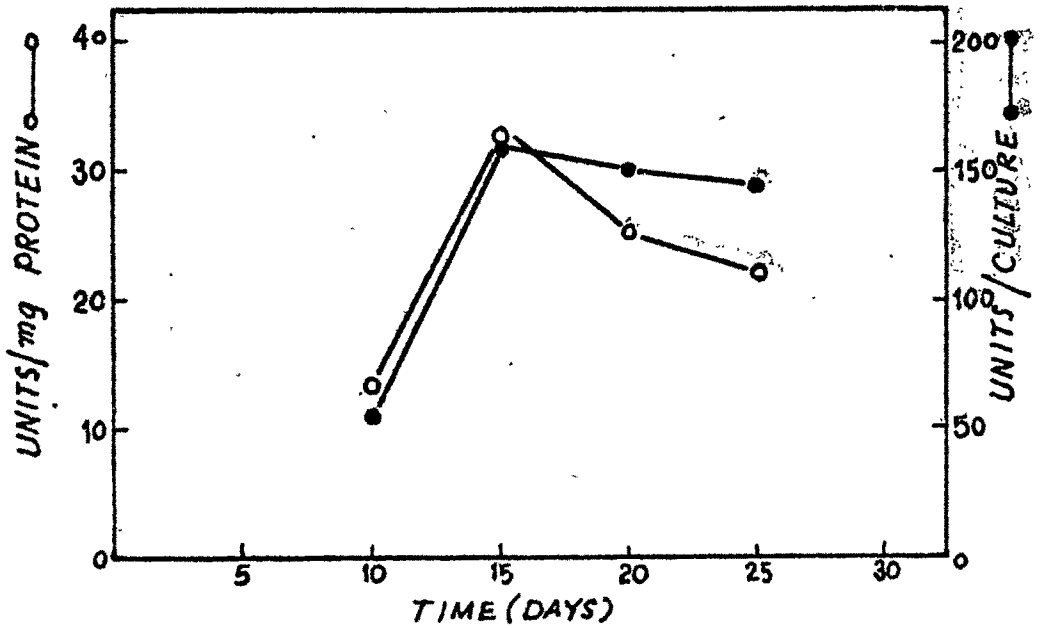

FIG. 37 - EFFECT OF 2 mM, L-TRYPTOPHAN ON TRYPTOPHAN SYNTHETASE ACTIVITY

Table - 26. Periodic changes in growth, alkaloid production and tryptophan synthetase activity in E. alsinoides suspension cultures with L-tryptophan levels.

Inoculum : 200±20 mg tissue by fresh weight (dry weight 11.08 mg) in 25 ml of MS medium containing 2% sucrose and supplemented with 2.0 mg/l 2,4-D and 0.4 mg/l kinetin and 5 mM L-tryptophan.

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

Days	Fresh weight (mg/cult.)	Dry weight (mg/cult.)	Alkaloid		Tryptophan synthetase	
			(%)	(µg/cult.)	(units/mg protein)	(units/cult.)
0	200.00 (±1.232)	11.08 (±0.125)	0.008	-	-	-
5	321.0 (±0.631)	15.6 (±0.549)	0.006	0.93	-	-
10	630.9 (±2.202)	54.9 (±0.543)	0.006	3.3	15.2	47.317
15	1117.6 (±2.234)	61.82 (±0.683)	0.012	7.4	15.9	83.227
20	1438.8 (±3.841)	65.5 (±0.819)	0.012	7.9	19.736	83.82
25	1465.3 (±3.703)	67.7 (±0.766)	0.016	10.8	16.47	121.895
30	1414.6 (±3.996)	64.3 (±0.738)	0.014	9.0	15.647	112.095

Data represents average of five replicates.

Figures in parenthesis represent standard error.

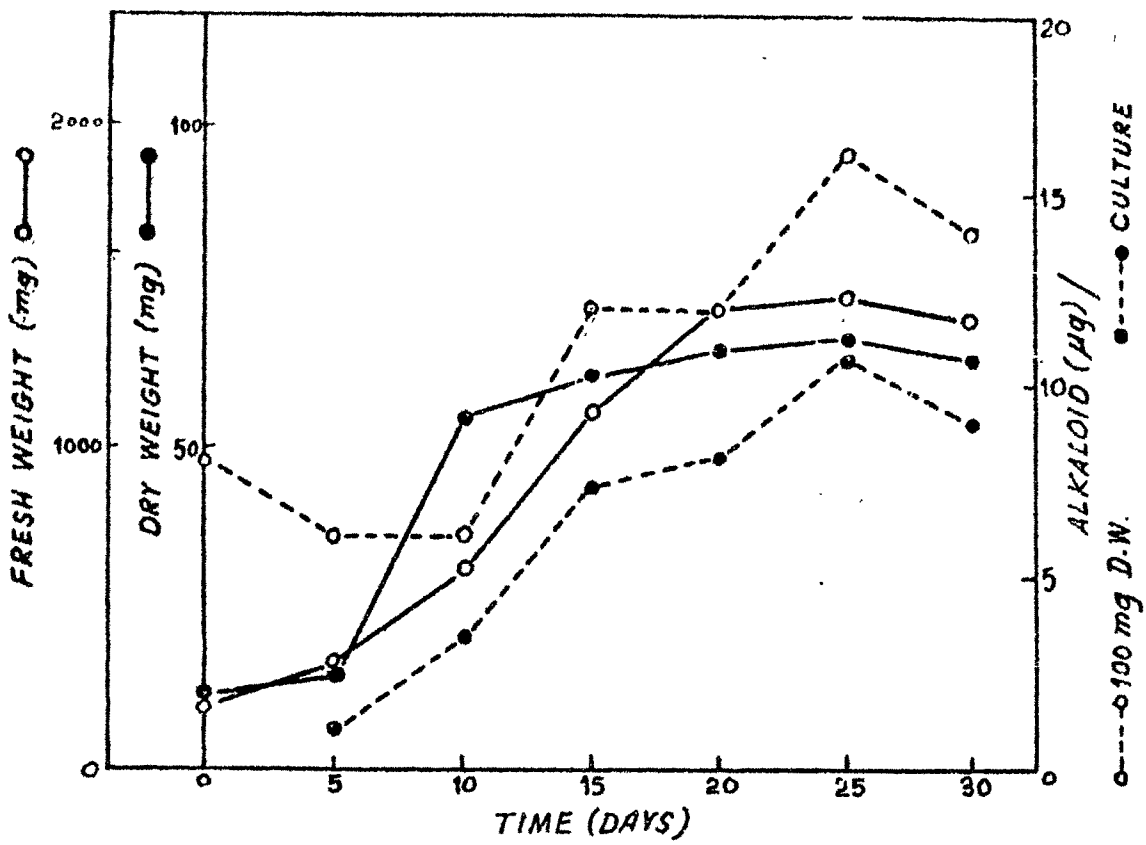

FIG.38-EFFECT OF 5 mM, L-TRYPTOPHAN ON GROWTH & ALKALOID PRODUCTION

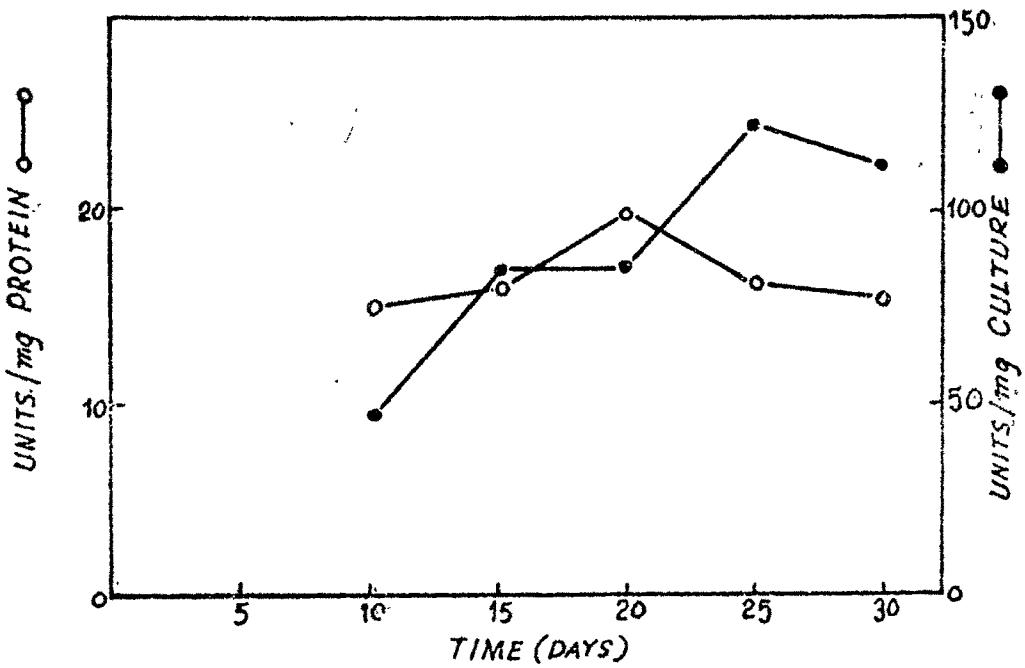

FIG.39-EFFECT OF 5 mM, L-TRYPTOPHAN ON TRYPTOPHAN SYNTHETASE ACTIVITY

Table - 27. Periodic changes in growth, alkaloid production and tryptophan synthetase activity in E. alsinoides suspension cultures with L-tryptophan levels.

Inoculum : 200±20 mg tissue by fresh weight (dry weight 11.08 mg) in 25 ml of MS medium containing 2% sucrose and supplemented with 2.0 mg/l 2,4-D and 0.4 mg/l kinetin and 10 mM L-tryptophan.

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

Days	Fresh weight (mg/cult.)	Dry weight (mg/cult.)	Alkaloid		Tryptophan synthetase	
			(%)	(µg/cult.)	(units/mg protein)	(units/cult.)
0	200.00 (± 1.232)	11.08 (±0.125)	0.008	-	-	--
5	315.0 (± 0.837)	15.4 (±0.344)	0.009	1.38	-	-
10	607.7 (± 1.676)	39.1 (±0.514)	0.009	3.5	15.5	60.946
15	968.2 (± 2.264)	53.7 (±0.622)	0.01	5.4	16.0	126.573
20	1125.1 (± 3.353)	59.1 (±0.809)	0.019	11.2	16.5	154.035
25	1230.9 (± 4.597)	65.2 (±0.984)	0.02	13.0	15.45	92.317
30	1220.8 (± 1.231)	64.1 (±0.8164)	0.19	12.0	-	-

Data represents average of five replicates.

Figures in parenthesis represent standard error.

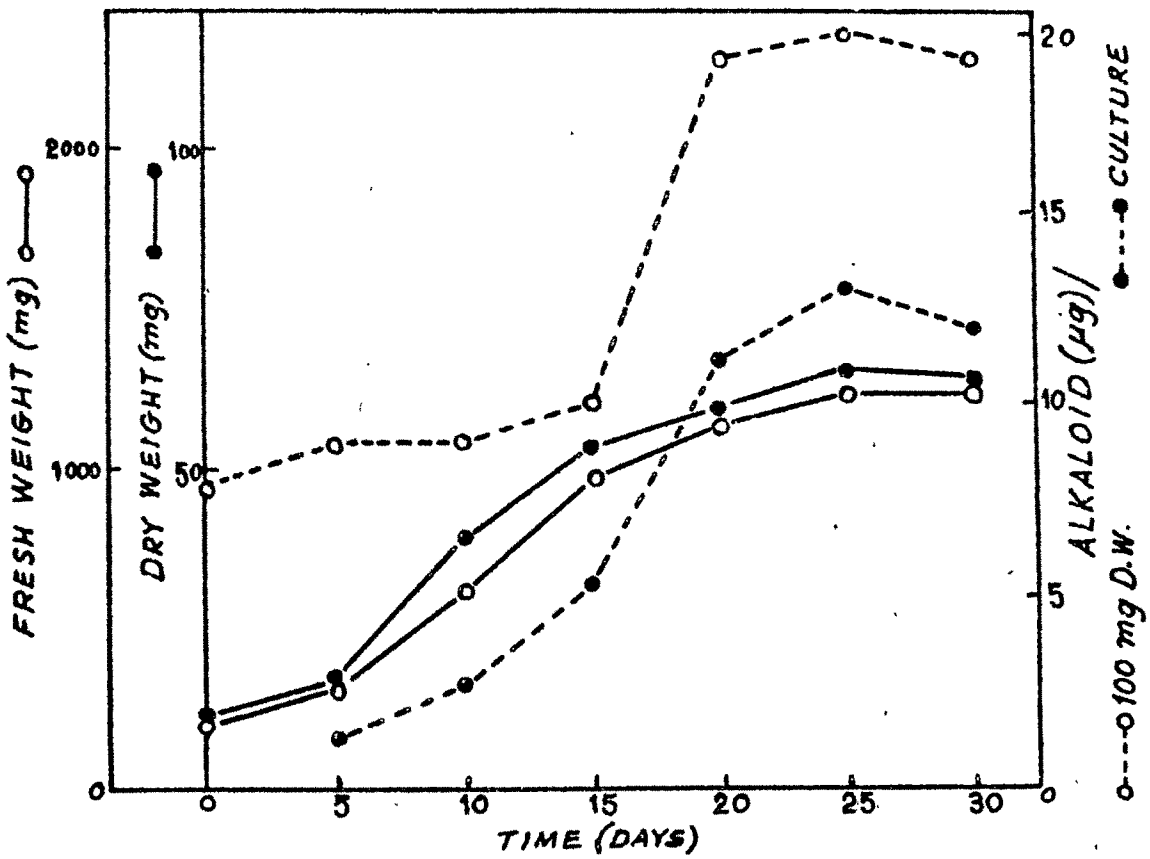

FIG. 40 - EFFECT OF 10 mM, L-TRYPTOPHAN ON GROWTH & ALKALOID PRODUCTION

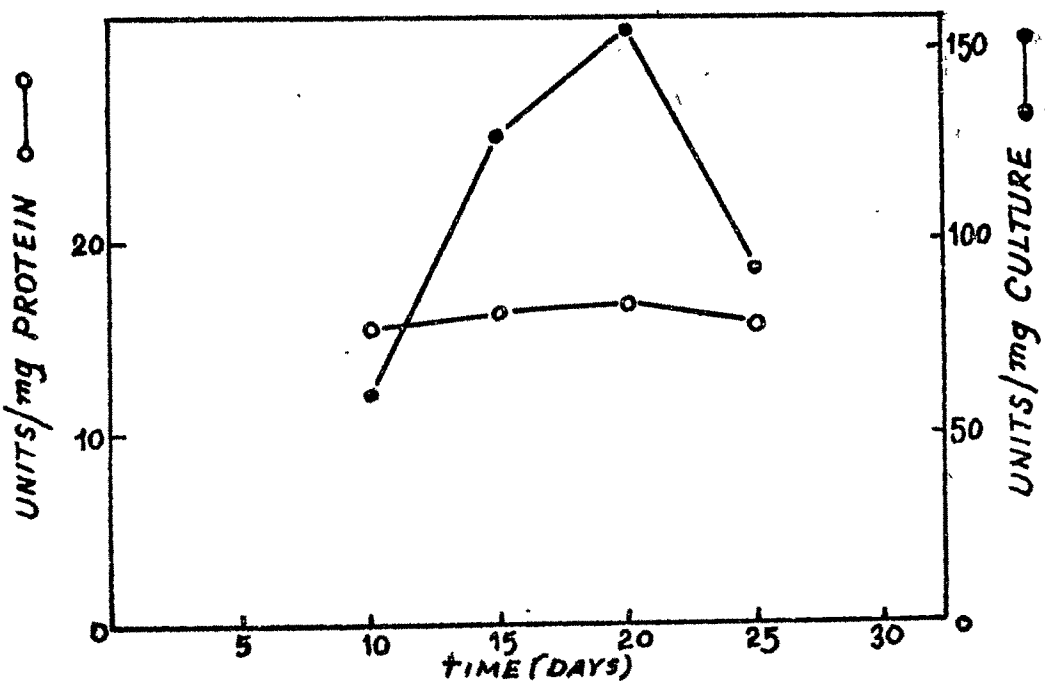

FIG. 41 - EFFECT OF 10 mM, L-TRYPTOPHAN ON TRYPTOPHAN SYNTHETASE ACTIVITY

Tryptophan (Table 25, Fig. 37) and 10 mM Tryptophan (Table 27, Fig. 41) tryptophan synthetase activity was not recorded on day 30. However, in 5 mM Tryptophan treatment, the enzyme activity was recorded from day 10 till day 30 (Table 26, Fig. 39). The tryptophan synthetase activity was highest on day 20 in all cases except in case of 2 mM Tryptophan treatment where the maximum activity was recorded on day 15.

I - 2 : Effect of periodic addition of Tryptophan on growth and alkaloid production.

L-tryptophan was incorporated in the medium at 2 mM and 10 mM levels on day 5, day 10, day 15 and day 20 of culture. The tissue was harvested for the measurement of growth and estimation of alkaloid production after 25 days in culture. The results are presented in Tables 28, 29 and Figs. 42, 43.

The growth of tissue was comparatively less than the control in cultures in which 2 mM Tryptophan was added periodically. The cultures where Tryptophan was added on day 20, showed comparatively more growth than in other treatments (Table 28, Fig. 42). In studies with 10 mM Tryptophan, the cultures where Tryptophan was incorporated on days 5 and 10 of culture, the growth of tissue was much reduced in comparison to other treatments when it was added later. Clearly, late addition of Tryptophan (2 mM as well as 10 mM) when most of the growth had already taken place, its adverse effect was not

Table - 28. Effect of early and late addition of Tryptophan on growth and alkaloid production in E. alsinoides suspension cultures.

Inoculum : 200±20 mg by fresh weight (dry weight 11.08 mg) in 25 ml of MS medium containing 2% sucrose and supplemented with 2.0 mg/l 2,4-D and 0.4 mg/l kinetin and 2 mM L-tryptophan.

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

TIME of addition (2 mM L-tryptophan)	Fresh weight (mg/cult.)	Dry weight (mg/cult.)	Alkaloid (%)	Alkaloid (µg/culture)
CONTROL	4084.25 (±11.212)	197,01 (±3.701)	0.012	23.856
5th day	3123.6 (±10.123)	120.08 (±2.321)	0.014	16.8
10th day	3186.5 (±10.765)	120.27 (±2.171)	0.014	16.8
15th day	3201.6 (±10.123)	120.18 (±2.561)	0.013	15.6
20th day	3415.7 (±11.216)	121.08 (±3.171)	0.12	14.52

Data represents average of five replicates.

Figures in parenthesis represent standard error.

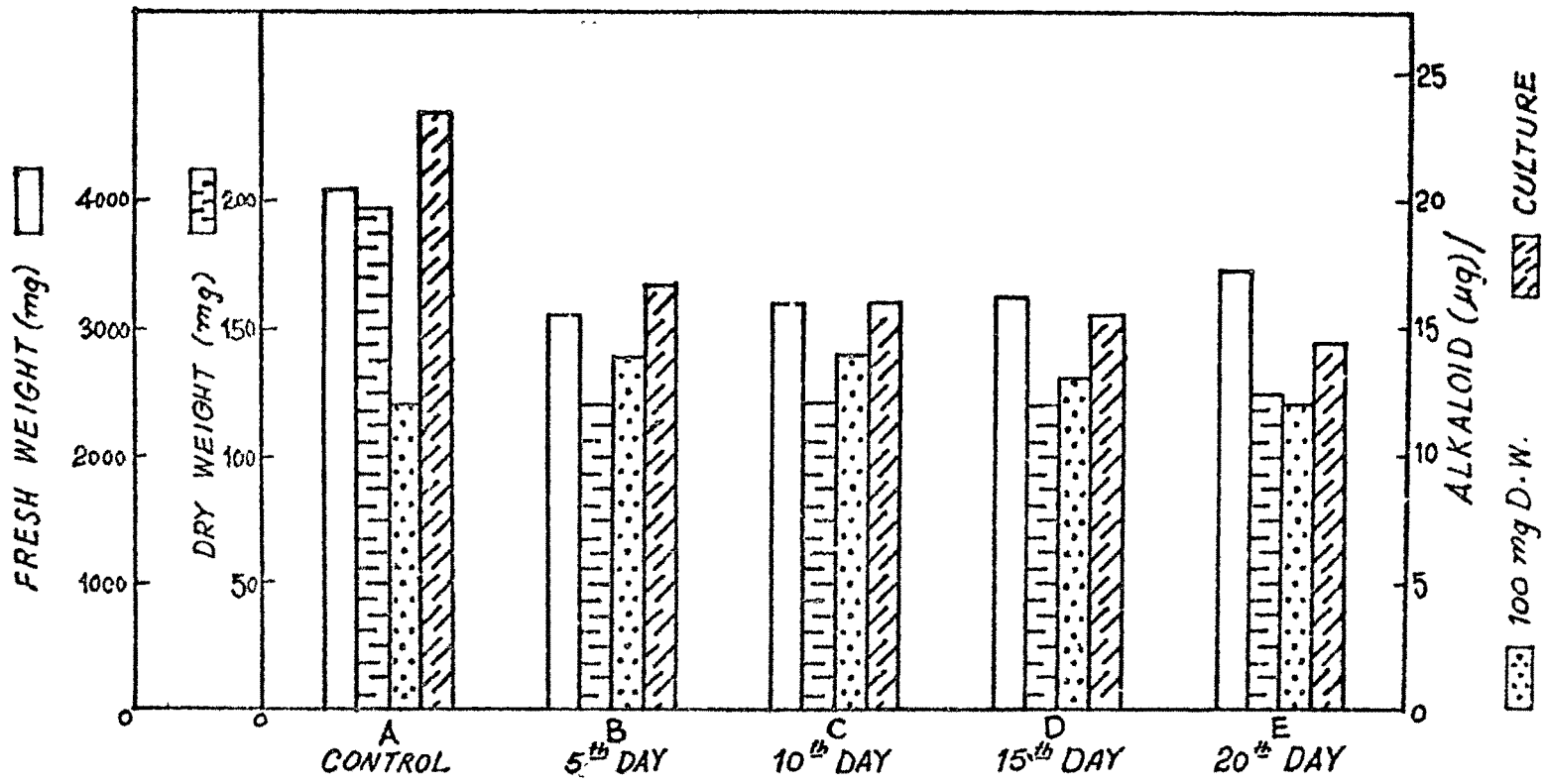

FIG. 42-EFFECT OF SEQUENTIAL ADDITION OF 2 mM, L-TRYPTOPHAN ON GROWTH AND ALKALOID PRODUCTION.

Table - 29. Effect of early and late addition of Tryptophan on growth and alkaloid production in E. alsinoides suspension cultures.

Inoculum : 200±20 mg by fresh weight (dry weight 11.08 mg) in 25 ml of MS medium containing 2% sucrose and supplemented with 2.0 mg/l 2,4-D and 0.4 mg/l kinetin and 10 mM L-tryptophan.

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

TIME of addition (10 mM L-tryptophan)	Fresh weight (mg/cult.)	Dry weight (mg/cult.)	Alkaloid	
			(%)	(µg/culture)
CONTROL	4084.25 (±11.212)	197.01 (±3.701)	0.012	23.856
5th day	2725.5 (± 8.123)	110.72 (±1.187)	0.02	22.1
10th day	2780.05 (± 7.625)	110.91 (±1.521)	0.021	23.28
15th day	3001.62 (± 9.213)	115.65 (±3.112)	0.018	20.8
20th day	3215.6 (± 7.164)	117.23 (±3.212)	0.016	18.75

Data represents average of five replicates.

Figures in parenthesis represent standard error.

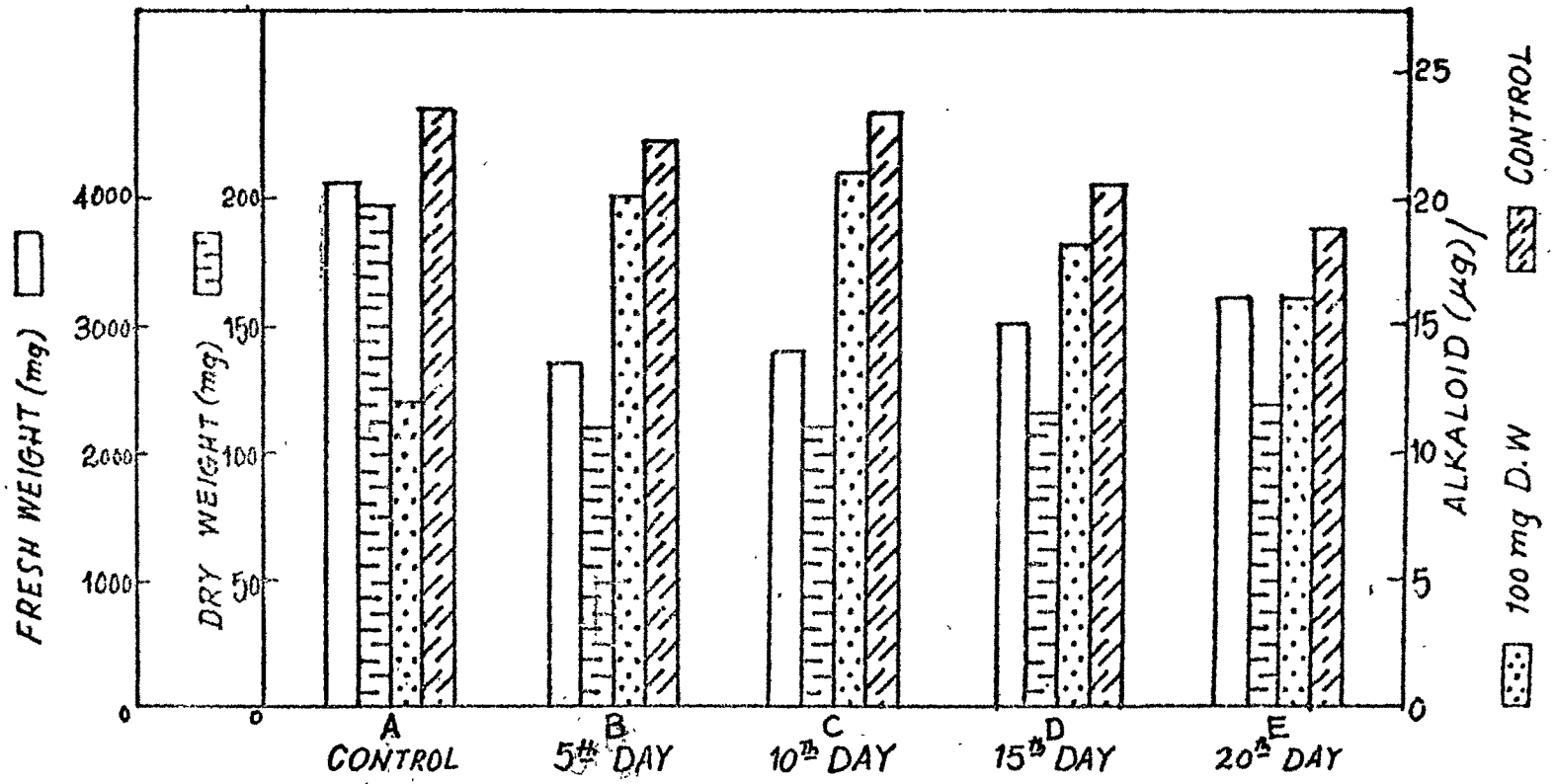

FIG. 43 - EFFECT OF SEQUENTIAL ADDITION OF 10 m M, L-TRYPTOPHAN ON GROWTH AND ALKALOID PRODUCTION

that severe. On the other hand, addition of Tryptophan on day 5 and day 10 when growth was just initiated, the suppression of growth was very pronounced, more so in presence of higher level of Tryptophan.

Unlike growth, early addition of Tryptophan appreciably enhanced alkaloid production (Tables 28 and 29, Figs. 42 and 43); however, later addition had less stimulatory effect on alkaloid production. Still, in the latter case, at higher Tryptophan concentration alkaloid content was significantly higher than in the control on day 25.

I - 3 : Effect of 5-methyl tryptophan on growth, alkaloid production and tryptophan synthetase activity.

The concentration of 5-methyl tryptophan administered was only 2 mM, because 5-methyl tryptophan was found to completely inhibit the growth of tissue. At the level tested, it showed a fresh and dry weight increase of about 3 fold. The alkaloid production was slightly enhanced compared to normal cultures (Table 30, Fig. 44).

Tryptophan synthetase activity was not detected till day 10 of cultures. Day 10 recorded maximum activity and then the enzyme activity declined. Day 20 recorded minimum activity and thereafter the enzyme activity was not detected (Table 30, Fig. 45).

Table - 30. Periodic changes in growth, alkaloid production and tryptophan synthetase activity in E. alsinoides suspension cultures at 2 mM, 5-methyl DL-Tryptophan.

Inoculum : 200±20 mg tissue by fresh weight (dry weight 11.01 mg) in 25 ml of MS medium containing 2% sucrose and supplemented with 2.0 mg/l 2,4-D and 0.4 mg/l kinetin and 2 mM, 5-methyl-DL-tryptophan.

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

Days	Fresh weight (mg/cult.)	Dry weight (mg/cult.)	Alkaloid (%)	Alkaloid (µg/cult.)	Tryptophan synthetase (units/mg protein)	Tryptophan synthetase (units/cult.)
0	200.00 (±1.517)	11.01 (±0.175)	0.008	-	-	-
5	429.6 (±0.286)	27.3 (±0.018)	0.006	1.6	-	-
10	527.3 (±0.358)	34.2 (±0.235)	0.007	2.4	23.53	42.18
15	528.2 (±0.317)	35.3 (±0.178)	0.007	2.47	20.0	42.25
20	585.0 (±0.413)	35.4 (±0.234)	0.01	3.5	3.0	5.85
25	597.6 (±0.856)	35.6 (±0.219)	0.013	4.6	-	-
30	488.3 (±0.541)	35.0 (±0.321)	0.012	4.2	-	-

Data represents average of five replicates.

Figures in parenthesis represent standard error.

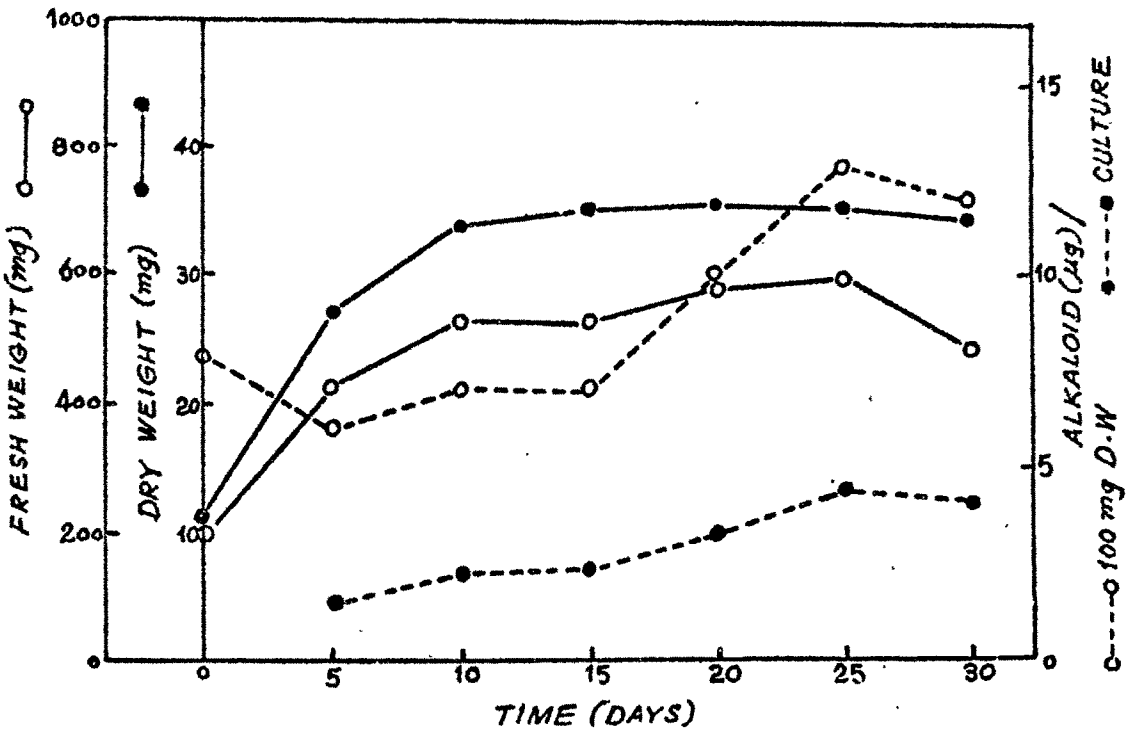

FIG.44-EFFECT OF 5-METHYL-TRYPTOPHEN ON GROWTH & ALKALOID PRODUCTION

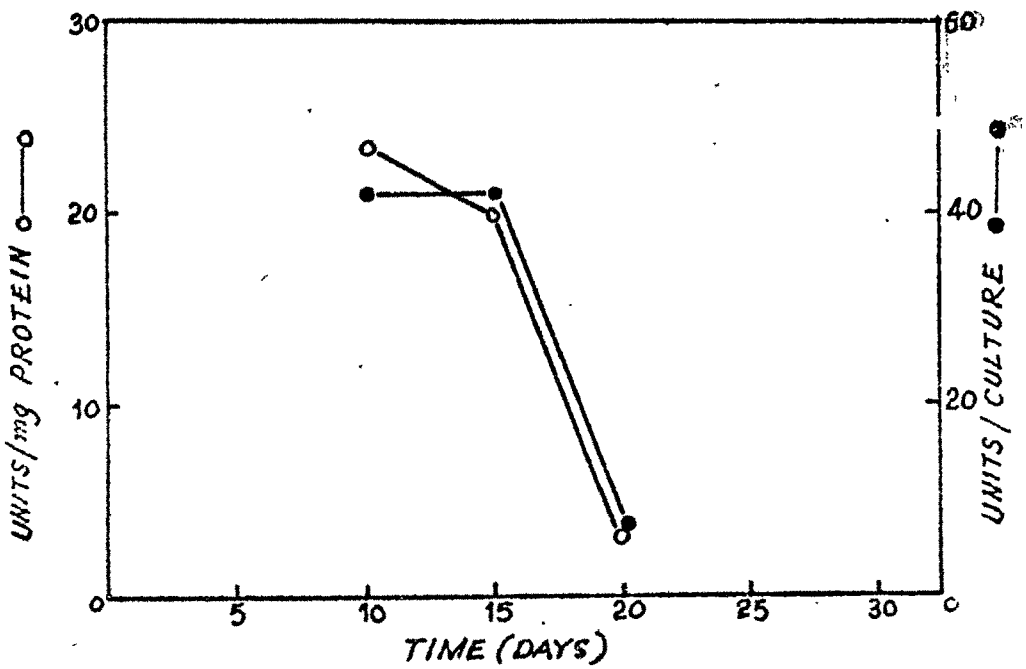

FIG.45-EFFECT OF 5-METHYL-TRYPTOPHEN ON TRYPTOPHAN SYNTHETASE ACTIVITY

I - 4 : Effect of DL-mevalonic acid on growth, alkaloid production and tryptophan synthetase activity.

The concentrations of Mevalonic acid incorporated in the medium were 2 mM and 5 mM. As in previous cases, measured aliquots of cell suspension weighing 200±20 mg by fresh weight were transferred to 25 ml of MS medium (supplemented with 2.0 mg/l 2,4-D, and 0.4 mg/l kinetin in addition to 2% sucrose) containing 2 or 5 mM Mevalonic acid. The culture vessels were incubated at 25±2°C in continuous light for 30 days. A fixed number of replicates was harvested at the interval of 5 days for the determination of growth, alkaloid production and enzyme assay. Results are presented in Tables 31, 32 and Figs. 46-49.

Mevalonic acid had a marked effect on growth of tissue. The tissue growth was comparatively more than the normal cultures at both the levels of Mevalonic acid tested. Cultures growing on 2 mM Mevalonic acid had a 20 fold increase in fresh weight and about 18 fold increase in dry weight (Table 31, Fig. 46). At 5 mM Mevalonic acid, the fresh weight as well as dry weight increase was more or less similar to cultures containing 2 mM Mevalonic acid (Table 32, Fig. 48). Alkaloid production was enhanced in both the concentrations tried, being about 50% higher than in the control (Tables 31, 32 and Figs. 46, 48).

Table - 31. Periodic changes in growth, alkaloid production and tryptophan synthetase activity in E. alsinoides suspension cultures with 2 mM, Mevalonic acid.

Inoculum : 200±20 mg tissue by fresh weight (dry weight 11.01 mg) in 25 ml of MS medium containing 2% sucrose and supplemented with 2.0 mg/l 2,4-D and 0.4 mg/l kinetin and 2 mM, Mevalonic acid.

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

Days	Fresh weight (mg/cult.)	Dry weight (mg/cult.)	Alkaloid		Tryptophan Synthetase	
			(%)	(ug/cult.)	(units/mg protein)	(units/cult.)
0	200.00 (± 1.517)	11.01 (±0.175)	0.008	-	-	-
5	312.5 (± 0.289)	16.8 (±0.193)	0.006	1.0	-	-
10	884.2 (± 0.858)	47.8 (±0.242)	0.006	2.9	11.66	30.96
15	1372.1 (± 2.316)	94.01 (±0.317)	0.009	8.5	12.57	36.65
20	2727.5 (± 2.413)	140.1 (±0.624)	0.011	15.4	14.84	40.91
25	4098.1 (± 7.252)	193.1 (±1.121)	0.015	28.9	-	-
30	4097.25 (± 7.125)	193.01 (±1.652)	0.014	27.0	-	-

Data represents average of five replicates.

Figures in parenthesis represent standard error.

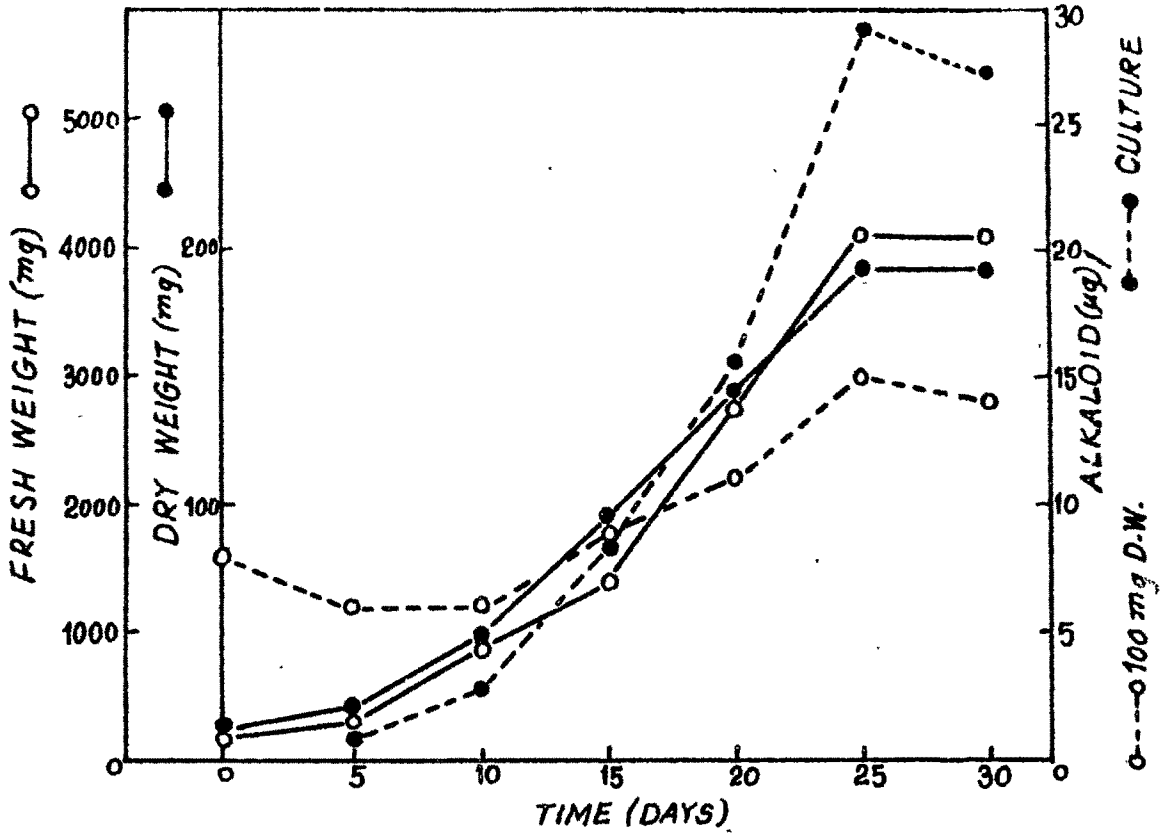

FIG. 46-EFFECT OF 2 mM MEVALONIC ACID ON GROWTH & ALKALOID PROD.

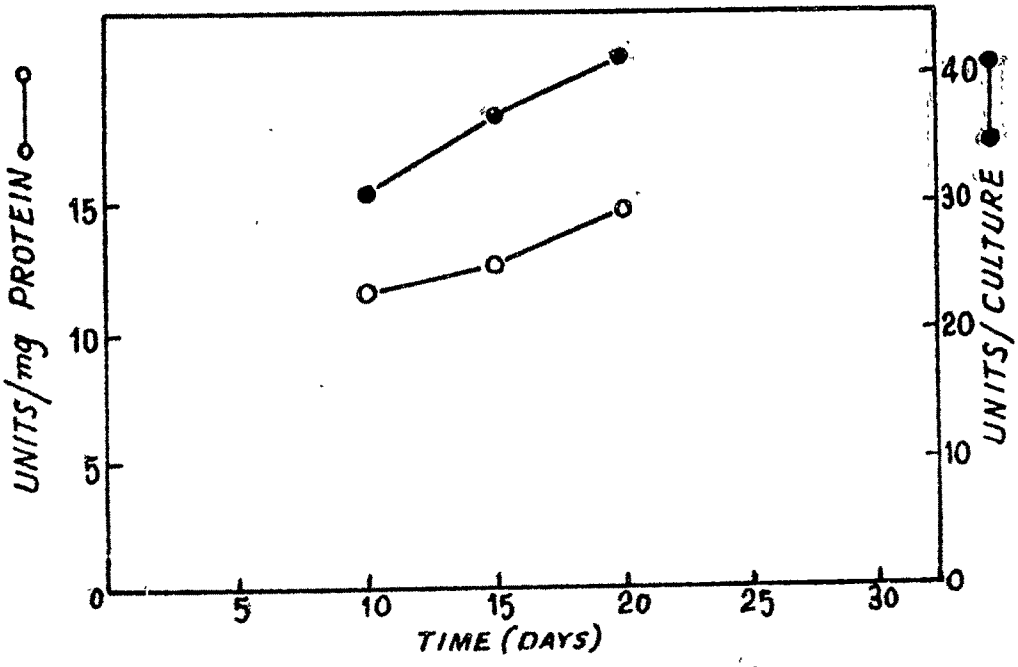

FIG. 47-EFFECT OF 2 mM MEVALONIC ACID ON TRYPTOPHAN SYNTHETASE ACTIVITY

Table - 32. Periodic changes in growth, alkaloid production and tryptophan synthetase activity in E. alsinoides suspension cultures with 5 mM, Mevalonic acid.

Inoculum : 200±20 mg tissue by fresh weight (dry weight 11.01 mg) in 25 ml of MS medium containing 2% sucrose and supplemented with 2.0 mg/l 2,4-D and 0.4 mg/l kinetin and 5 mM, Mevalonic acid.

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

Days	Fresh weight (mg/cult.)	Dry weight (mg/cult.)	Alkaloid		Tryptophan Synthetase	
			(%)	(µg/cult.)	(units/mg protein)	(Units/cult.)
0	200.00 (±1.517)	11.01 (±0.175)	0.008	-	-	-
5	325.04 (±0.687)	15.78 (±0.186)	0.006	0.95	-	-
10	900.61 (±0.862)	48.27 (±0.321)	0.006	2.59	12.06	32.15
15	1576.01 (±1.236)	96.28 (±0.423)	0.009	8.6	14.74	48.26
20	2912.16 (± 3.642)	143.01 (±0.812)	0.012	17.2	16.13	68.01
25	4127.06 (± 5.216)	193.45 (±1.871)	0.016	31.0	-	-
30	4125.12 (± 7.123)	193.01 (±2.178)	0.013	25.1	-	-

Data represents average of five replicates.

Figures in parenthesis represent standard error.

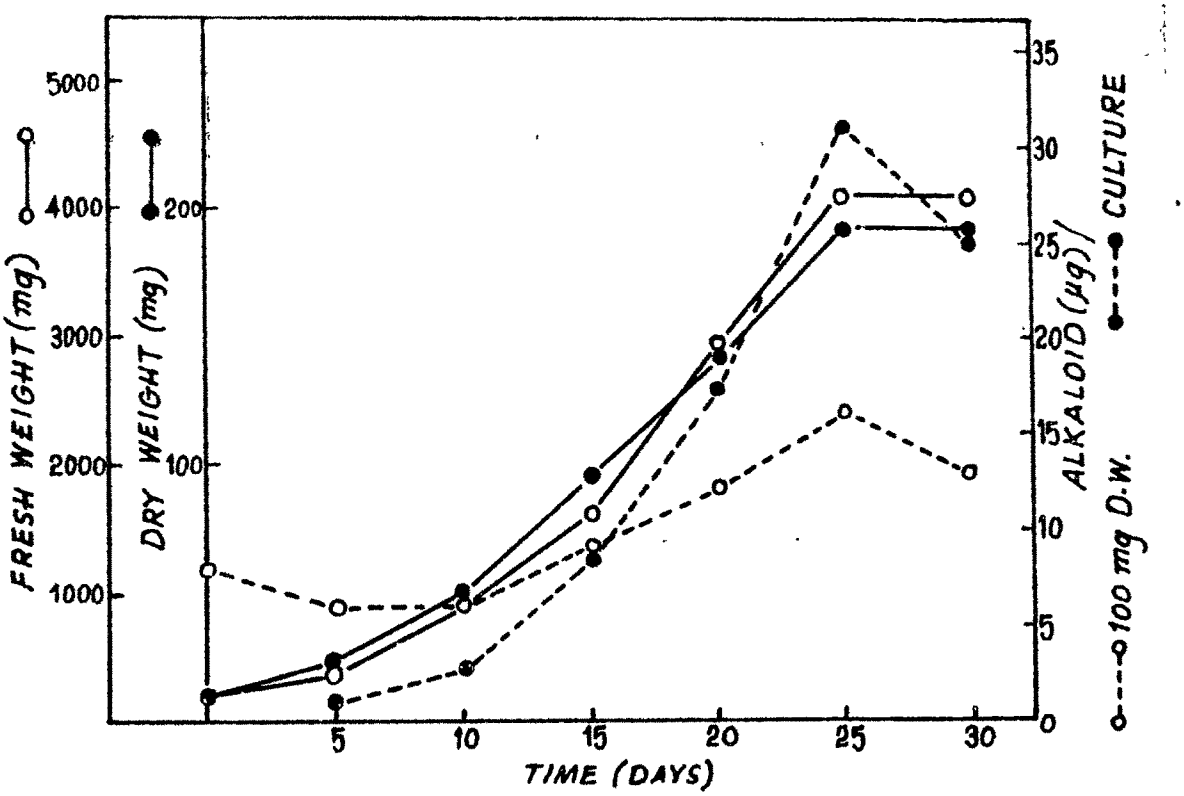

FIG. 48-EFFECT OF 5mM MEVALONIC ACID ON GROWTH & ALKALOID PROD.

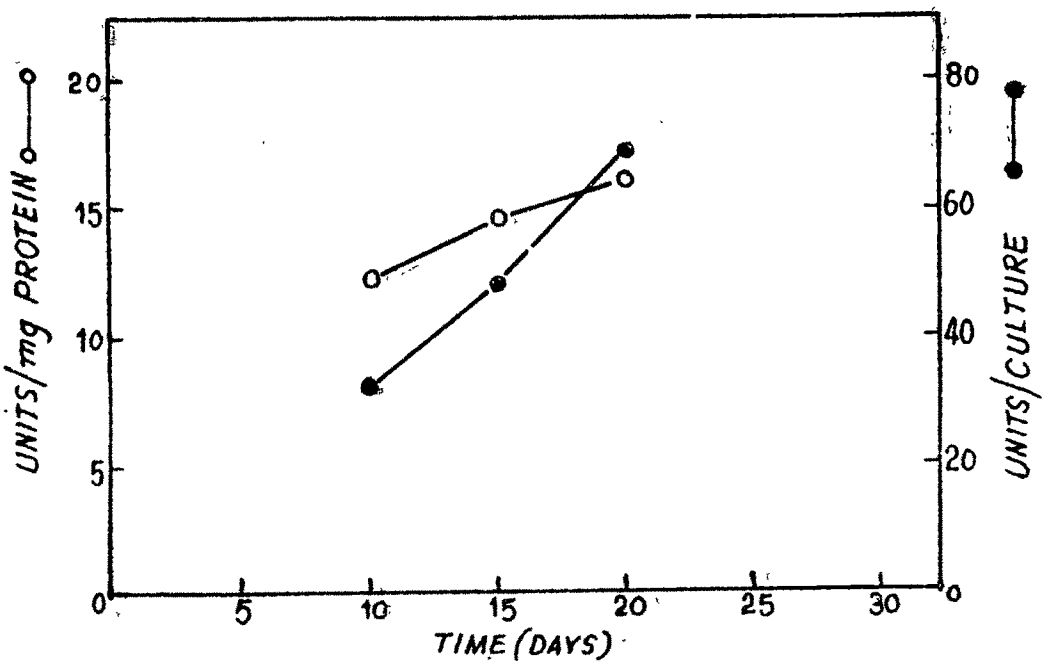

FIG. 49-EFFECT OF 5mM MEVALONIC ACID ON TRYPTOPHAN SYNTHETASE ACTIVITY

Enzyme activity was not detected in cultures on day 5 in presence of both 2 mM and 5 mM levels of Mevalonic acid. From day 10 of culture, the enzyme activity steadily increased in both the treatments, showing maximum activity on day 20. No enzyme activity was recorded on day 25 and day 30. Specific activity of tryptophan synthetase expressed per milligram protein was slightly higher in cultures containing 5 mM Mevalonic acid (Table 32, Fig. 49) than 2 mM Mevalonic acid (Table 31, Fig. 47).

I - 5 : Effect of Methionine on growth, alkaloid production and tryptophan synthetase activity.

Effect of Methionine was examined only at 2 mM concentration, as it inhibited growth at higher concentrations. Methionine at 2 mM, did not have any stimulatory effect on alkaloid production. On the other hand, growth of tissue was inhibited showing only about 2.5 fold increase in fresh weight and dry weight on day 25 (Table 33, Fig. 50).

As in the previous experiments, enzyme activity was not detected on day 5, but it steadily increased from day 10 to day 20 of culture. Peak enzyme activity was registered on day 20, (Table 33, Fig. 51) after which the enzyme activity was not detected.

Table - 33. Periodic changes in growth, alkaloid production and tryptophan synthetase activity in *E. alsinoides* suspension cultures with 2 mM, Methionine.

Inoculum : 200±20 mg tissue by fresh weight (dry weight 11.01 mg) in 25 ml of MS medium containing 2% sucrose and supplemented with 2.0 mg/l 2,4-D and 0.4 mg/l kinetin and 2 mM, Methionine.

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

Days	Fresh weight (mg/cult.)	Dry weight (mg/cult.)	Alkaloid (%)	Alkaloid (µg/cult.)	Tryptophan Synthetase (units/mg protein)	Tryptophan Synthetase (units/cult.)
0	200.00 (±1.517)	11.01 (±0.175)	0.008	-	-	-
5	419.06 (±0.716)	26.73 (±0.321)	0.006	1.6	-	-
10	487.12 (±0.912)	27.81 (±0.234)	0.006	1.66	13.25	41.84
15	491.213 (±0.812)	28.01 (±0.123)	0.007	1.96	15.125	61.24
20	501.136 (±1.212)	29.235 (±0.145)	0.01	2.9	17.231	75.123
25	502.161 (±1.816)	29.212 (±0.512)	0.012	3.5	-	-
30	501.896 (±1.716)	29.181 (±0.613)	0.012	3.5	-	-

Data represents average of five replicates.

Figures in parenthesis represent standard error.

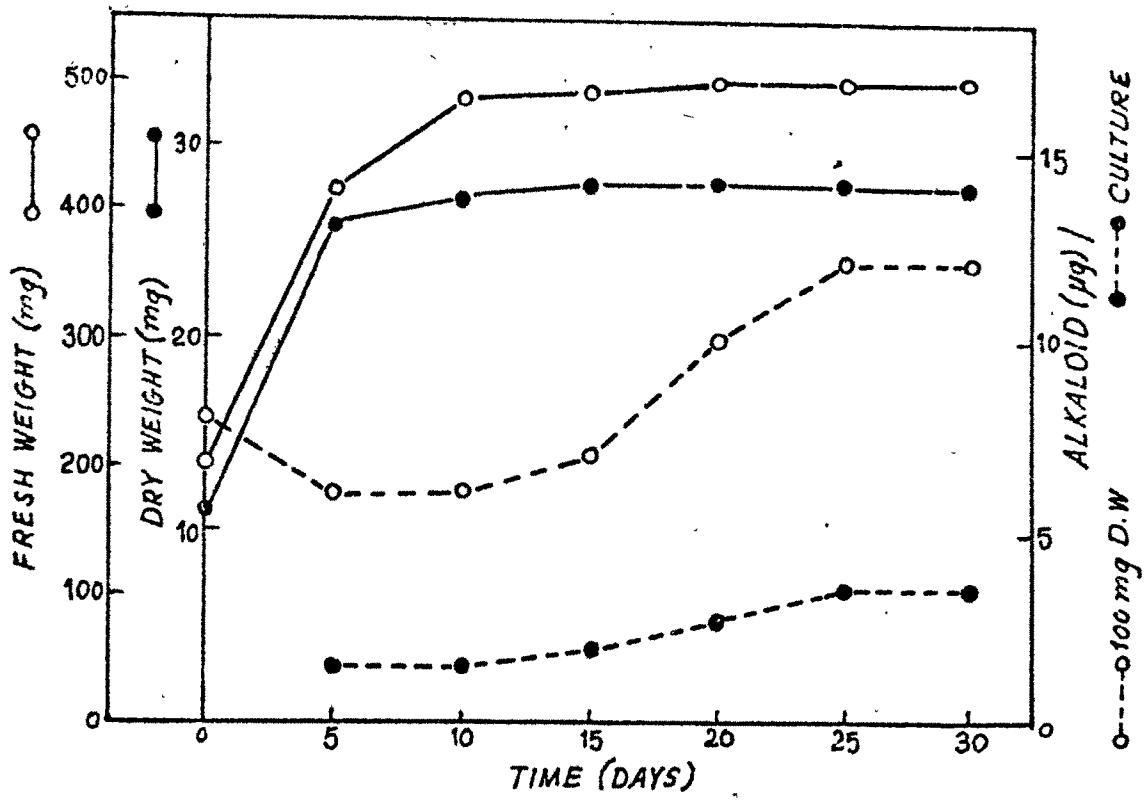

FIG. 50 - EFFECT OF METHIONINE ON GROWTH & ALKALOID PRODUCTION

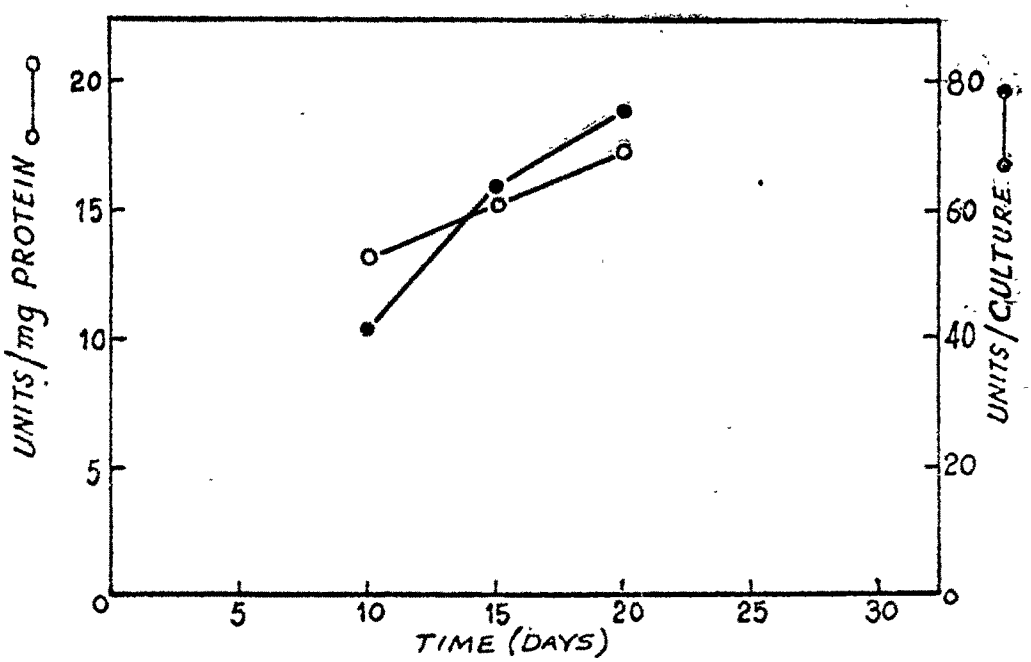

FIG. 51 - EFFECT OF METHIONINE ON TRYPTOPHAN SYNTHETASE ACTIVITY

I - 6 : Effect of Anthranilic acid on growth, alkaloid production and tryptophan synthetase activity.

Anthranilic acid levels incorporated in the medium were 1 mM and 2 mM. It was found to have an adverse effect on growth of tissue as well as alkaloid production. The cultures growing in 1 mM Anthranilic acid showed only 2 fold increase in fresh weight and about 1.5 fold increase in dry weight (Table 34, Fig. 52). The alkaloid production was also far less compared to normal cultures. At the highest level tested (2 mM) the growth of tissue and alkaloid production were completely inhibited (Table 35, Fig. 54).

Similar to growth and alkaloid production, enzyme activity was also inhibited by Anthranilic acid. At 1 mM concentration the enzyme activity was detected at very low levels only. There was no activity on day 5. Day 15 recorded maximum activity and then it declined as on day 20 and further the activity was not detected (Table 34, Fig. 53). At 2 mM concentration the enzyme activity was completely inhibited (Table 35).

I - 7 : Effect of Indole on growth, alkaloid production and tryptophan synthetase activity.

To find out the role of Indole in alkaloid production, it was incorporated in the medium at 0.5 mM and 2 mM concentrations. At 0.5 mM, the growth of tissue was inhibited.

Table - 34. Periodic changes in growth, alkaloid production and tryptophan synthetase activity in E. alsinoides suspension cultures at 1 mM Anthranilic acid.

Inoculum : 200±20 mg tissue by fresh weight (dry weight 11.08 mg) in 25 ml of MS medium containing 2% sucrose and supplemented with 2.0 mg/l 2,4-D and 0.4 mg/l kinetin and 1 mM, Anthranilic acid.

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

Days	Fresh weight (mg/cult.)	Dry weight (mg/cult.)	Alkaloid		Tryptophan Synthetase	
			(%)	(µg/cult.)	(units/mg protein)	(units/cult.)
0	200.00 (±1.232)	11.08 (±0.125)	0.007	-	-	-
5	265.2 (±0.511)	14.01 (±0.126)	0.006	0.84	-	-
10	267.02 (±1.278)	14.21 (±0.123)	0.005	0.71	5.13	3.23
15	310.13 (±4.263)	16.81 (±0.231)	0.005	0.8	5.78	3.83
20	401.03 (±6.563)	18.1 (±0.625)	0.004	0.7	3.23	1.94
25	402.12 (±5.545)	18.21 (±0.459)	0.004	0.7	-	-
30	401.13 (±6.945)	18.01 (±1.023)	0.004	0.7	-	-

Data represents average of five replicates.

Figures in parenthesis represent standard error.

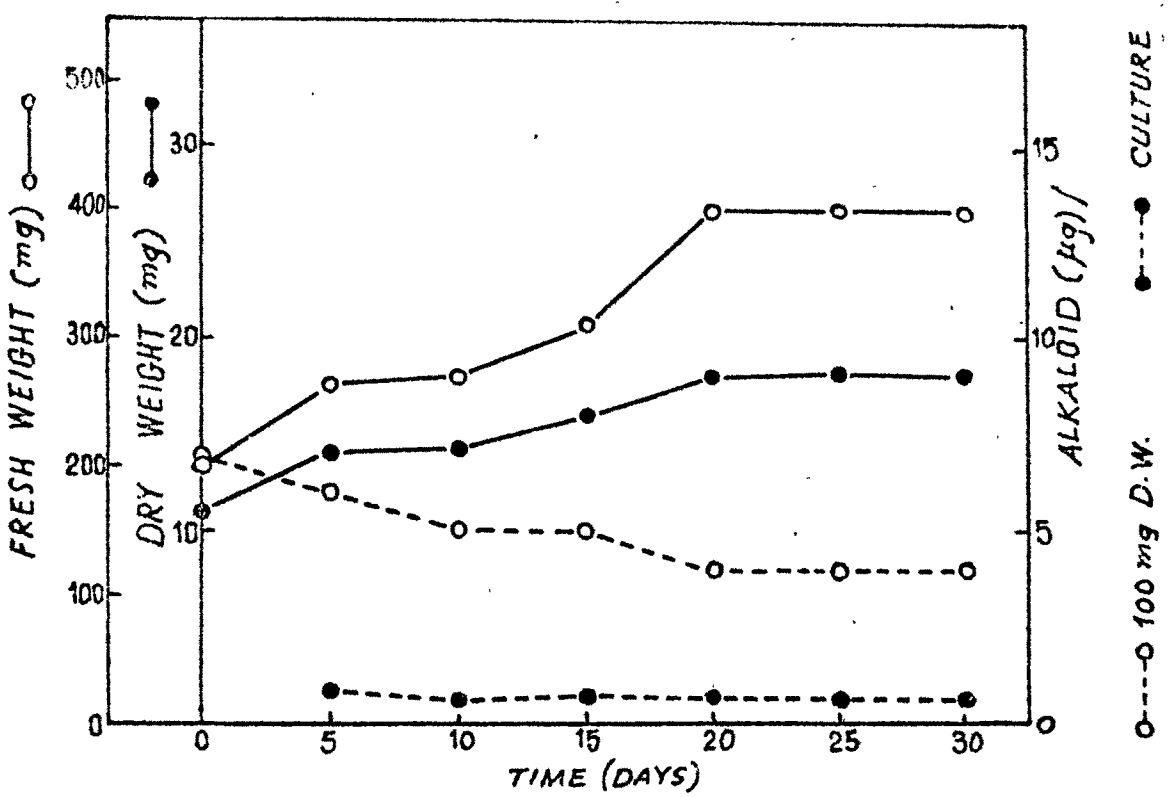

FIG.52-EFFECT OF ANTHRANILIC ACID ON GROWTH & ALKALOID PRODUCTION (1mM)

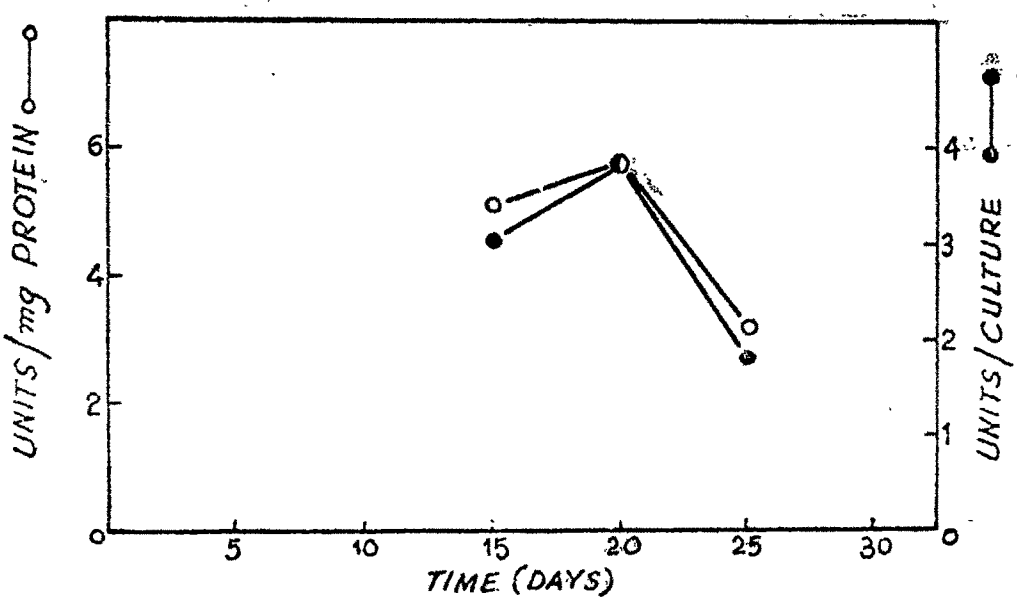

FIG.53-EFFECT OF 1mM ANTHRANILIC ACID ON TRYPTOPHAN SYNTHETASE - ACTIVITY.

Table - 35. Periodic changes in growth, alkaloid production and tryptophan synthetase activity in E. alsinoides suspension cultures at 2 mM Anthranilic acid.

Inoculum : 200±20 mg tissue by fresh weight (dry weight 11.08 mg) in 25 ml of MS medium containing 2% sucrose and supplemented with 2.0 mg/l 2,4-D and 0.4 mg/l kinetin and 2 mM, Anthranilic acid.

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

Days	Fresh weight (mg/cult.)	Dry weight (mg/cult.)	Alkaloid		Tryptophan Synthetase	
			(%)	(µg/cult.)	(units/ mg protein)	(units/cult.)
0	200.00 (±1.232)	11.08 (±0.125)	0.007	-	-	-
5	232.5 (±1.746)	14.1 (±0.637)	0.0025	0.35	-	-
10	232.12 (±1.065)	14.01 (±0.762)	0.0025	0.35	-	-
15	240.6 (±1.965)	14.5 (±0.752)	0.002	0.29	-	-
20	240.3 (±1.987)	14.2 (±0.769)	0.002	0.28	-	-
25	231.13 (±3.539)	14.1 (±1.213)	0.002	0.28	-	-
30	231.01 (±1.137)	14.02 (±0.798)	0.002	0.28	-	-

Data represents average of five replicates.

Figures in parenthesis represent standard error.

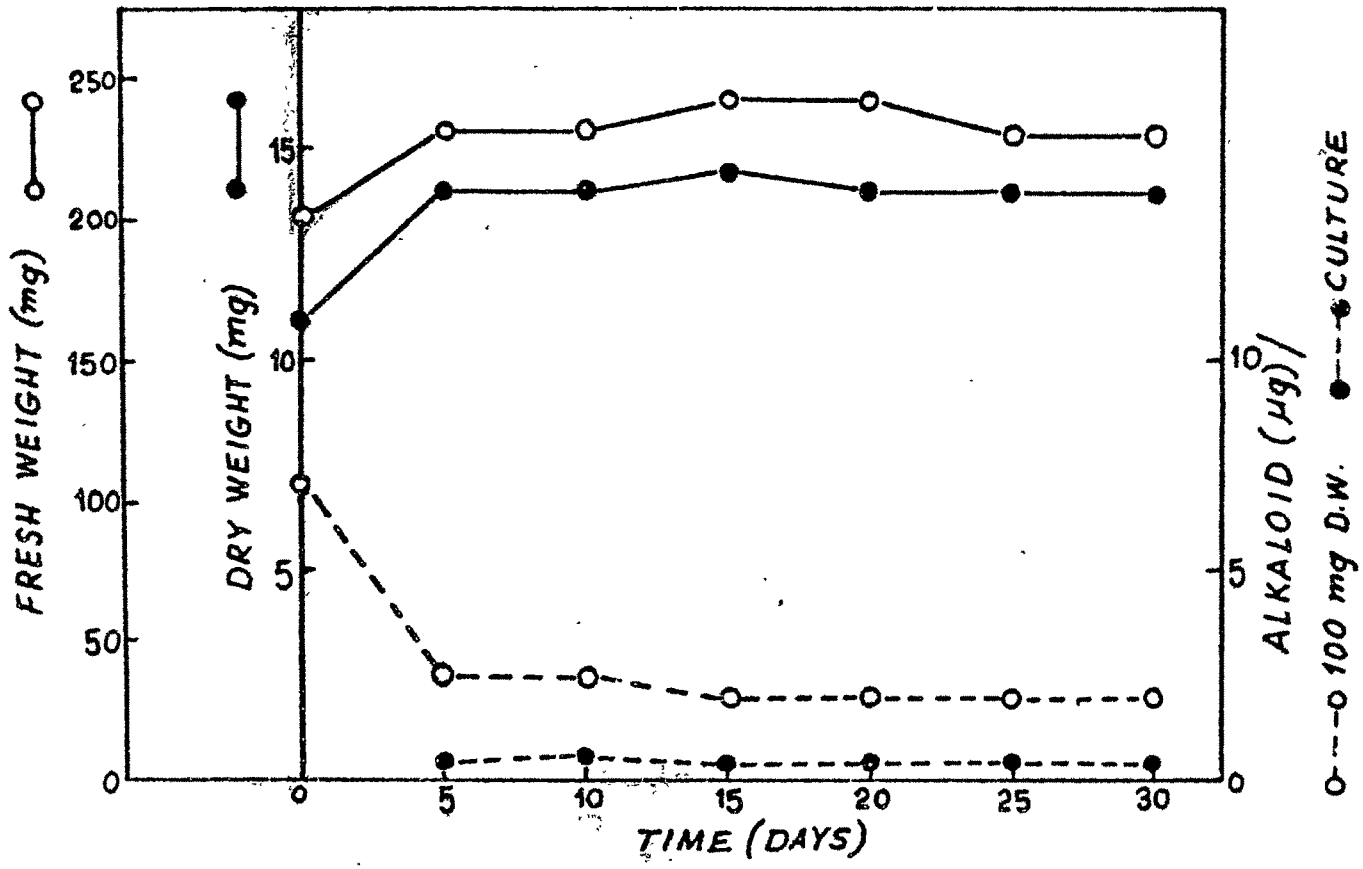

FIG.54-EFFECT OF 2mM ANTHRANILIC ACID ON GROWTH & ALKALOID PRODUCTION

The tissue did not synthesize alkaloid but there was a depletion in the levels of alkaloid present on day zero (Table 36, Fig. 55). At 2 mM concentration of Indole, the growth of tissue was completely inhibited. The tissue did not grow except for a slight increase in fresh weight on day 5. Similarly there was no enhancement in synthesis of alkaloid (Table 37, Fig. 57). Moreover, there was complete degradation of alkaloids initially present. The tissue became dark brown and hard at both the levels of Indole tested.

In cultures growing in 0.5 mM Indole the enzyme activity was not detected till day 10 and further it remained steady till day 15 (Table 36, Fig. 56). Day 20 recorded maximum activity and then the activity was not detected on days 25 and 30. At higher levels (2 mM) of Indole, the enzyme activity was very low and steady till day 20 and thereafter the activity was not detected (Table 37, Fig. 58).

I - 8 : Effect of DL-serine on growth, alkaloid and tryptophan synthetase activity.

Serine was incorporated in the medium at 2 mM and 5 mM levels. At the lower concentration of Serine studied, the fresh weight increase was about 18 fold and dry weight increase was about 16 fold. Alkaloid production also had a promotory effect than normal cultures (Table 38, Fig. 59). At the higher level of Serine tested (5 mM) the growth of tissue

Table - 36. Periodic changes in growth, alkaloid production and tryptophan synthetase activity in E. alsinoides suspension cultures at 0.5 mM Indole.

Inoculum : 200±20 mg tissue by fresh weight (dry weight 11.08 mg) in 25 ml of MS medium containing 2% sucrose and supplemented with 2.0 mg/l 2,4-D and 0.4 mg/l kinetin and 0.5 mM Indole.

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

Days	Fresh weight (mg/cult.)	Dry weight (mg/cult.)	Alkaloid (%)	Alkaloid (µg/cult.)	Tryptophan Synthetase (units/mg protein)	Tryptophan Synthetase (units/cult.)
0	200.00 (±1.232)	11.08 (±0.125)	0.007	-	-	-
5	269.2 (±3.136)	14.5 (±0.634)	0.003	0.44	-	-
10	270.4 (±1.612)	14.9 (±0.524)	0.003	0.43	27.7	13.27
15	270.5 (±1.851)	15.4 (±0.125)	0.003	0.46	27.7	13.27
20	270.5 (±2.216)	15.5 (±1.643)	0.003	0.46	34.4	13.52
25	257.5 (±2.561)	15.1 (±0.643)	0.003	0.45	-	-
30	236.4 (±2.042)	14.7 (±0.421)	0.003	0.44	-	-

Data represents average of five replicates.

Figures in parenthesis represent standard error.

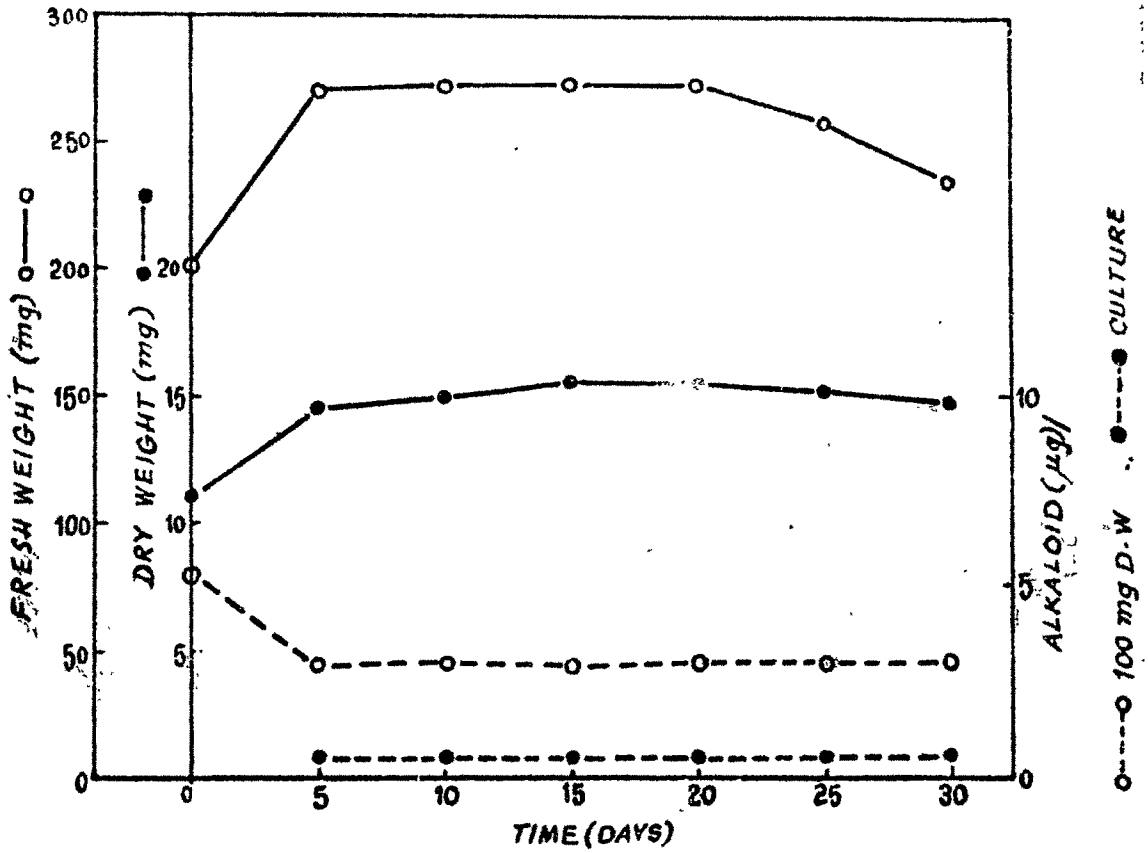

FIG. 55 - EFFECT OF 0·5 m M, INDOLE ON GROWTH & ALKALOID PRODUCTION

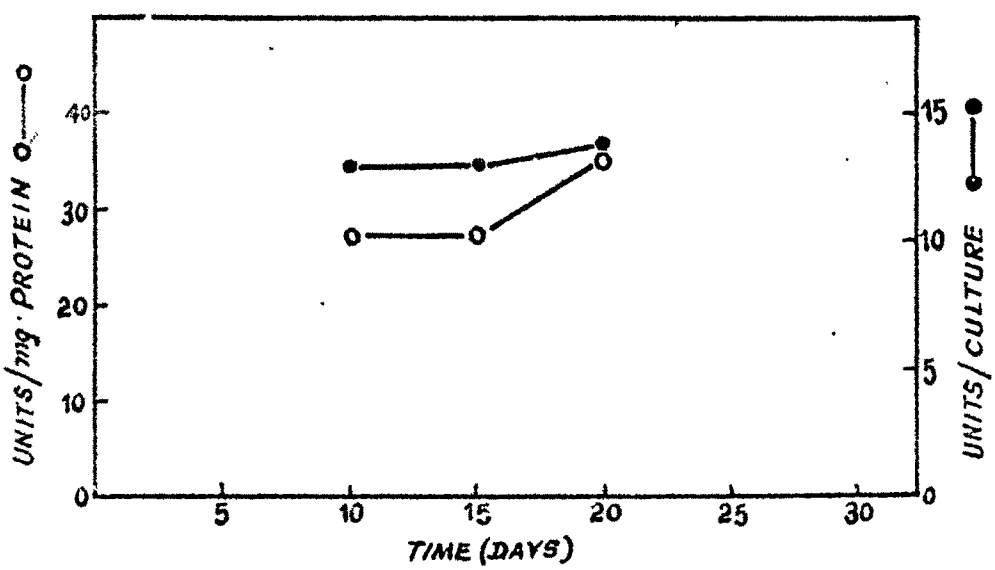

FIG. 56 - EFFECT OF 0·5 m M, INDOLE ON TRYPTOPHAN SYNTHETASE ACTIVITY

Table - 37. Periodic changes in growth, alkaloid production and tryptophan synthetase activity in E. alsinoides suspension cultures at 2 mM Indole.

Inoculum : 200±20 mg tissue by fresh weight (dry weight 11.08 mg) in 25 ml of MS medium containing 2% sucrose and supplemented with 2.0 mg/l 2,4-D and 0.4 mg/l kinetin and 2 mM Indole.

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

Days	Fresh weight (mg/cult.)	Dry weight (mg/cult.)	Alkaloid (%)	Alkaloid (µg/cult.)	Tryptophan Synthetase (units/mg protein)	Tryptophan Synthetase (units/cult.)
0	200.00 (±1.232)	11.08 (±0.125)	0.007	-	-	-
5	235.2 (±0.632)	14.3 (±0.214)	0.002	0.29	-	-
10	231.2 (±0.541)	14.3 (±0.213)	0.002	0.29	6.6	4.7
15	234.5 (±2.361)	14.5 (±0.752)	0.002	0.29	6.6	4.6
20	241.5 (±2.125)	14.7 (±0.135)	0.002	0.29	6.6	4.6
25	232.6 (±1.632)	14.2 (±0.761)	0.002	0.28	-	-
30	231.5 (±0.812)	14.1 (±0.302)	0.002	0.28	-	-

Data represents average of five replicates.

Figures in parenthesis represent standard error.

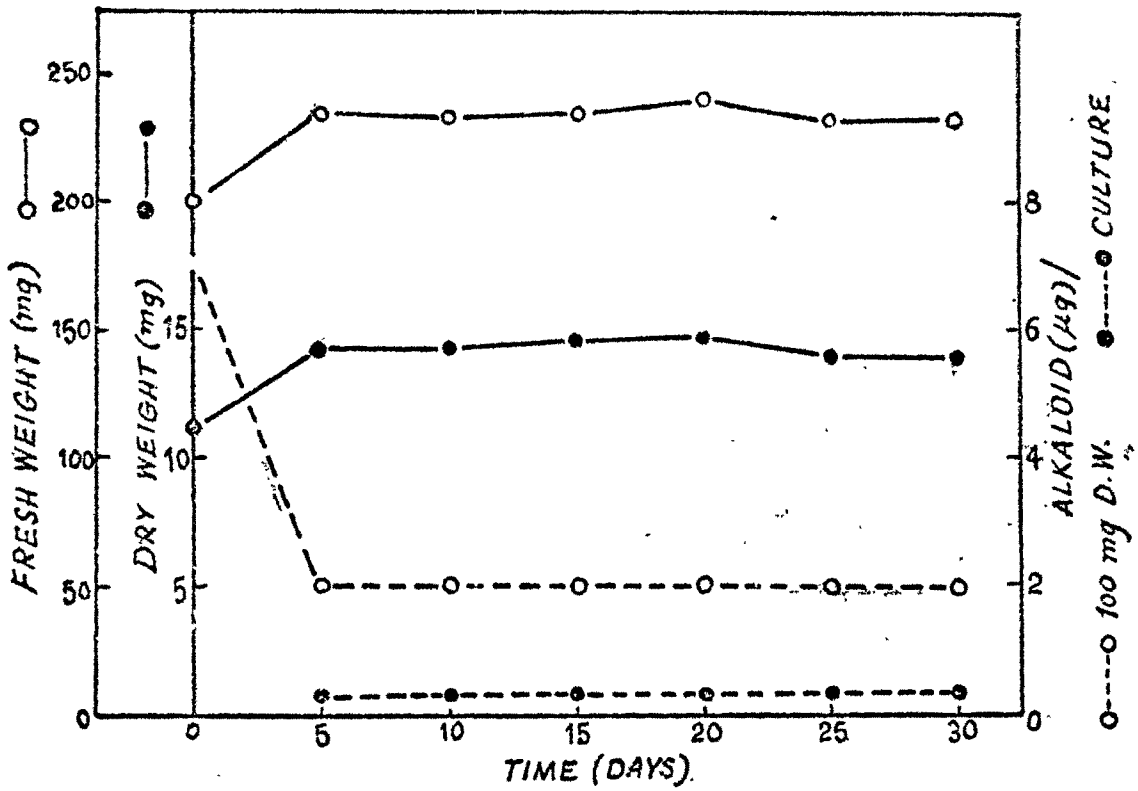

FIG. 57 - EFFECT OF 2 mM. INDOLE ON GROWTH & ALKALOID PRODUCTION

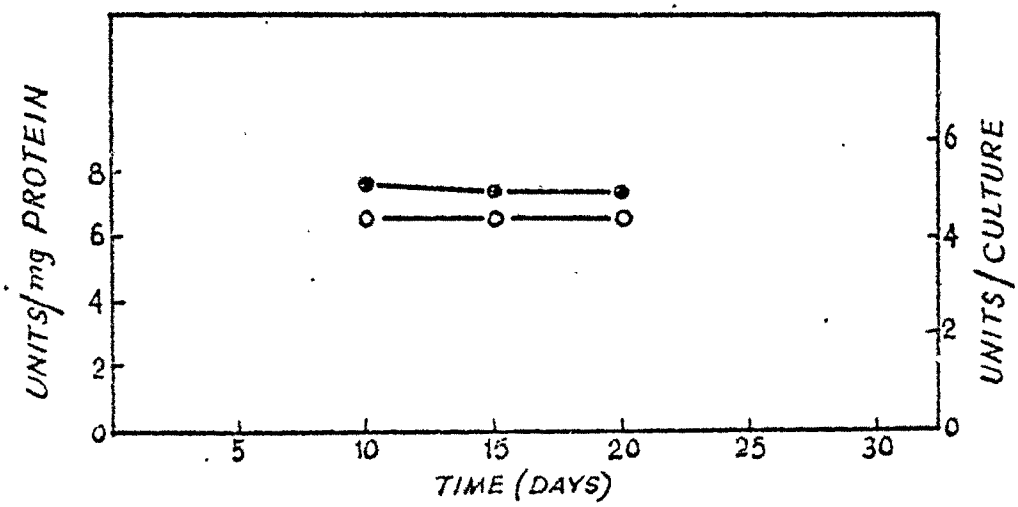

FIG. 58 - EFFECT OF 2 mM. INDOLE ON TRYPTOPHAN SYNTHETASE ACTIVITY

Table - 38. Periodic changes in growth, alkaloid production and tryptophan synthetase activity in *E. alsinoides* suspension cultures at 2 mM DL-serine.

Inoculum : 200±20 mg tissue by fresh weight (dry weight 11.08 mg) in 25 ml of MS medium containing 2% sucrose and supplemented with 2.0 mg/l 2,4-D and 0.4 mg/l kinetin and 2 mM DL-serine.

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

Days	Fresh weight (mg/cult.)	Dry weight (mg/cult.)	Alkaloid		Tryptophan Synthetase	
			(%)	(µg/cult.)	(units/mg protein)	(units/cult.)
0	200.00 (±1.232)	11.08 (±0.125)	0.007	-	-	-
5	511.5 (±2.56)	35.2 (±1.042)	0.006	2.1	-	-
10	874.3 (±1.106)	46.3 (±0.204)	0.006	2.8	20.128	57.03
15.	1372.21 (±5.545)	93.04 (±1.231)	0.008	7.4	25.01	126.71
20	2727.13 (±6.271)	138.01 (±1.321)	0.01	14.0	39.61	173.32
25	3703.01 (±4.232)	178.14 (±1.512)	0.014	25.0	33.11	156.12
30	3703.13 (±3.123)	178.01 (±0.785)	0.013	23.0	-	-

Data represents average of five replicates.

Figures in parenthesis represent standard error.

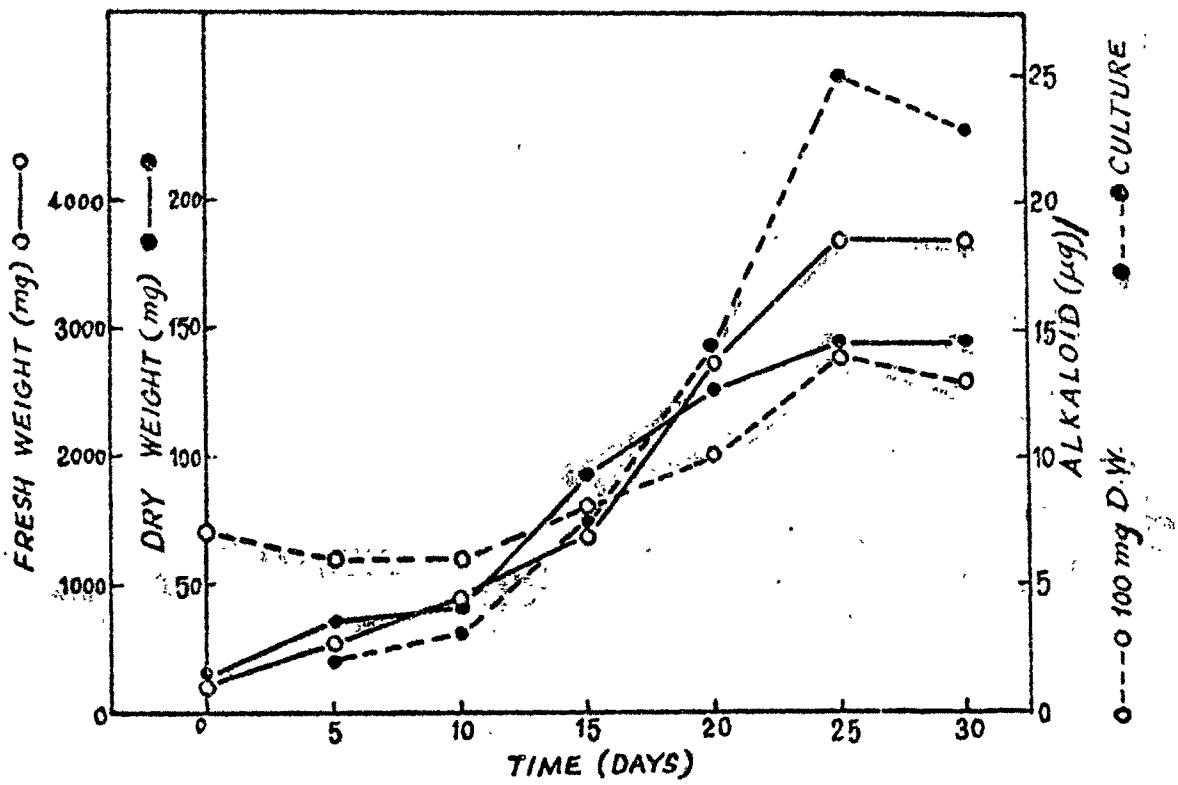

FIG. 59 - EFFECT OF 2 mM, DL-SERINE ON GROWTH & ALKALOID PRODUCTION

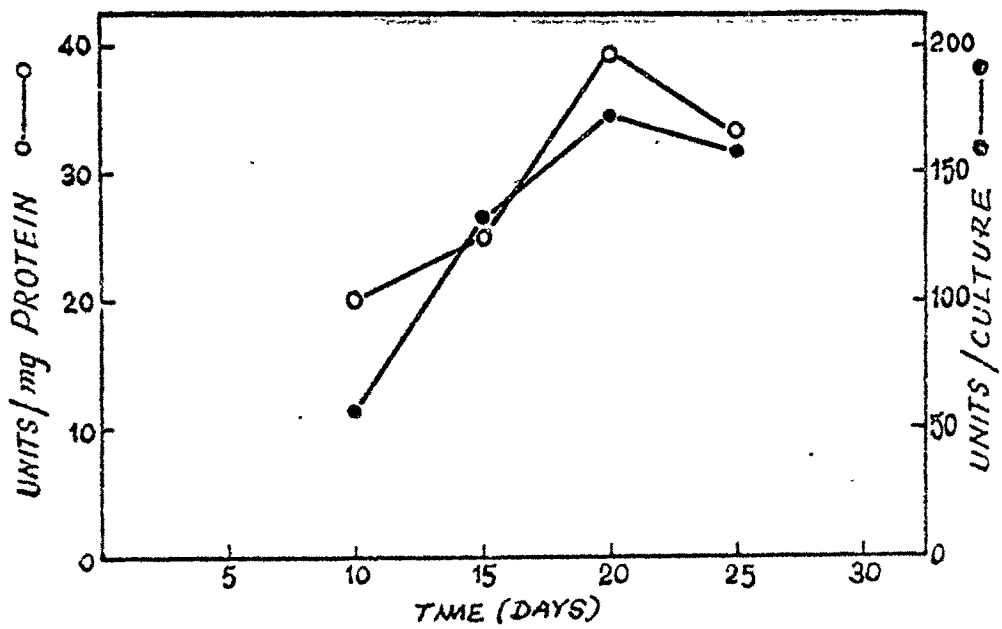

FIG. 60 - EFFECT OF 2 mM. DL-SERINE ON TRYPTOPHAN SYNTHETASE ACTIVITY

and alkaloid production showed a similar pattern as in the case of 2 mM Serine (Table 39, Fig. 61).

In cultures containing 2 mM Serine, enzyme activity was not detected till day 10, after which it steadily increased until day 20. Further the enzyme activity declined showing no activity on day 30 (Table 38, Fig. 60). A similar pattern of enzyme activity was observed in case of 5 mM Serine also (Table 39, Fig. 62).

I - 9 : Effect of DL-Serine + Indole on growth, alkaloid production and tryptophan synthetase activity.

To find out the effect of Serine in combination with Indole on growth and alkaloid production, 0.5 mM Indole + 2 mM Serine was incorporated in the medium.

The growth of tissue as well as alkaloid production was inhibited by Indole, Serine combination (Table 40, Fig. 63). The fresh weight and dry weight increase was only about 2.5 fold, whereas alkaloid production was far lower than in the control.

Enzyme activity was not detected till day 10. Then it increased steadily till day 20, when the maximum activity was attained (Table 40, Fig. 64). Later, the activity declined; days 25 and 30 showing no activity.

Table - 39. Periodic changes in growth, alkaloid production and tryptophan synthetase activity in *E. alsinoides* suspension cultures at 5 mM DL-serine.

Inoculum : 200±20 mg tissue by fresh weight (dry weight 11.08 mg) in 25 ml of MS medium containing 2% sucrose and supplemented with 2.0 mg/l 2,4-D and 0.4 mg/l kinetin and 5 mM DL-serine.

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

Days	Fresh weight (mg/cult.)	Dry weight (mg/cult.)	Alkaloid		Tryptophan Synthetase	
			(%)	(µg/cult.)	(units/mg protein)	(units/cult.)
0	200.00 (±1.232)	11.08 (±0.125)	0.007	-	-	-
5	509.4 (±2.123)	35.01 (±1.046)	0.006	2.1	-	-
10	901.17 (±2.375)	49.02 (±1.212)	0.006	2.9	17.231	59.01
15	1481.01 (±4.361)	93.62 (±2.167)	0.008	7.5	22.01	125.67
20	2812.361 (±5.632)	137.68 (±3.156)	0.009	12.39	37.521	171.17
25	3783.123 (±7.123)	178.67 (±2.167)	0.015	27.8	33.81	148.023
30	3780.621 (±7.267)	178.01 (±3.231)	0.014	24.9	-	-

Data represents average of five replicates.

Figures in parenthesis represent standard error.

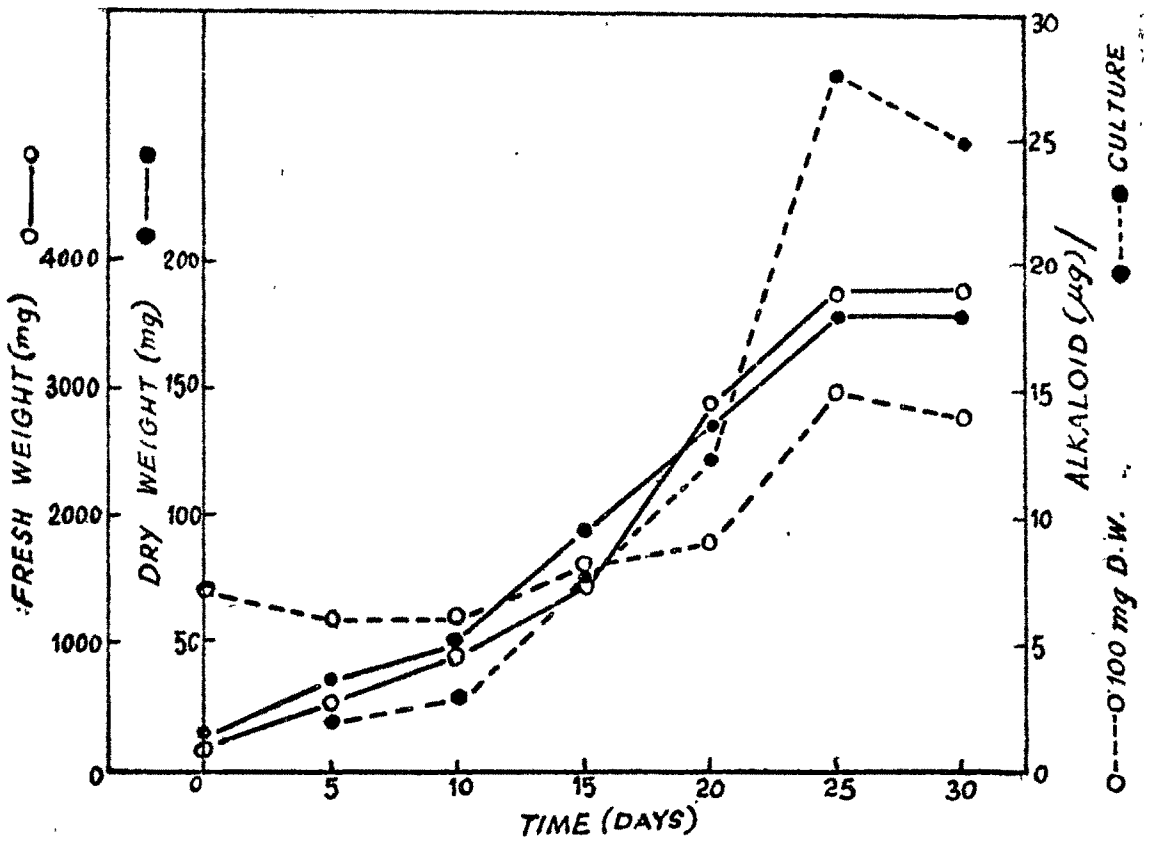

FIG. 61 - EFFECT OF 5 m M DL-SERINE ON GROWTH & ALKALOID PRODUCTION

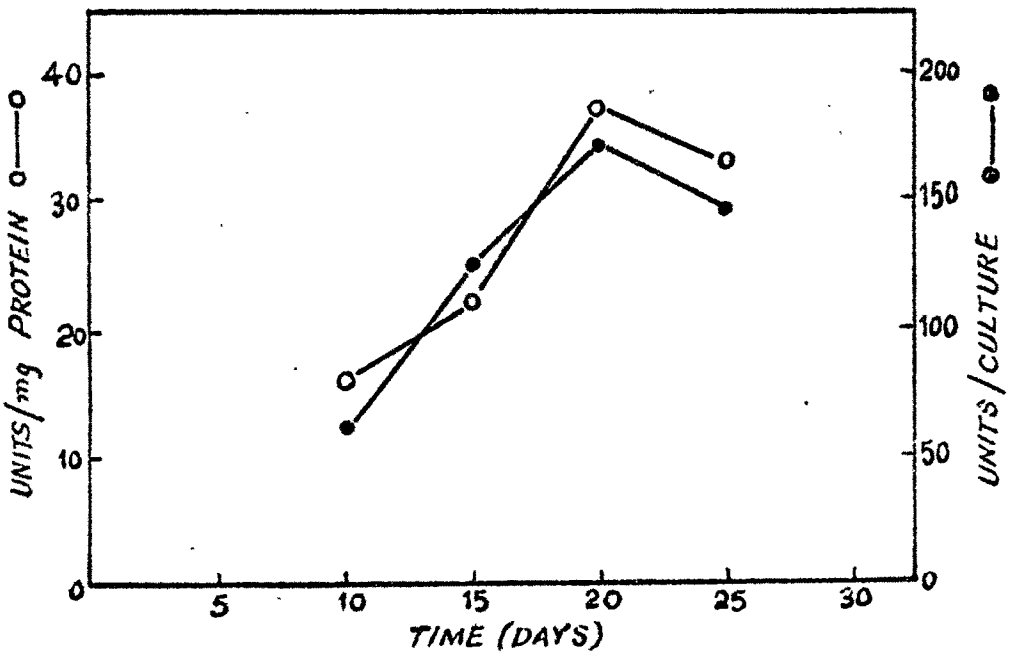

FIG. 62 - EFFECT OF 5 m M DL-SERINE ON TRYPTOPHAN SYNTHETASE ACTIVITY

Table - 40. Periodic changes in growth, alkaloid production and tryptophan synthetase activity in E. alsinoides suspension cultures in DL-serine + Indole levels.

Inoculum : 200±20 mg tissue by fresh weight (dry weight 11.17 mg) in 25 ml of MS medium containing 2% sucrose and supplemented with 2.0 mg/l 2,4-D and 0.4 mg/l kinetin and 2mM DL-serine + 0.5 mM, Indole.

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

Days	Fresh weight (mg/cult.)	Dry weight (mg/cult.)	Alkaloid (%)	Alkaloid (µg/cult.)	Tryptophan Synthetase (units/mg protein)	Tryptophan Synthetase (units/cult.)
0	200.00 (±1.367)	11.17 (±0.817)	0.007	-	-	-
5	269.2 (±1.326)	14.2 (±0.251)	0.005	0.7	-	-
10	269.4 (±1.81)	14.3 (±0.432)	0.005	0.7	5.12	4.12
15	281.5 (±2.123)	15.6 (±0.151)	0.006	0.9	9.13	7.37
20	321.2 (±3.621)	17.1 (±0.752)	0.006	1.03	5.76	4.81
25	512.3 (±5.123)	28.3 (±1.213)	0.006	1.7	-	-
30	513.1 (±5.361)	28.1 (±1.461)	0.006	1.7	-	-

Data represents average of five replicates.

Figures in parenthesis represent standard error.

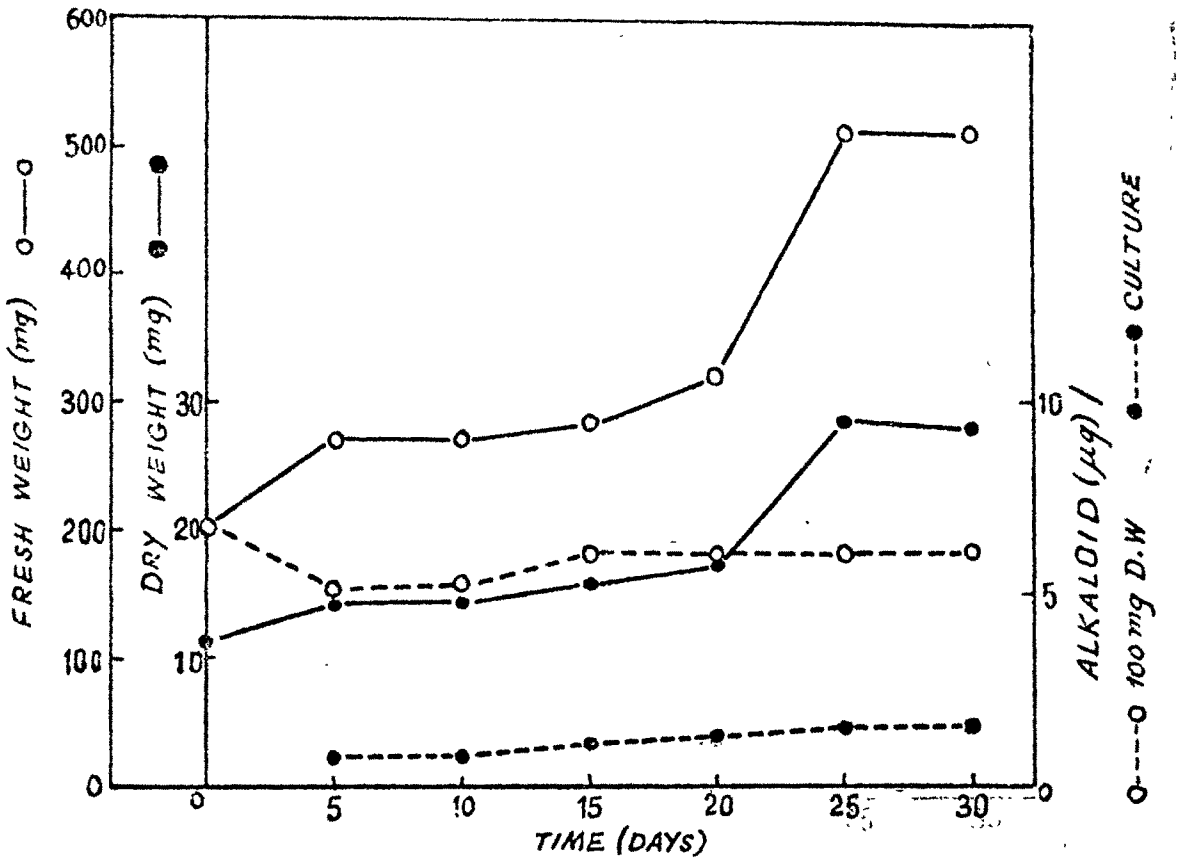

FIG. 63 - EFFECT OF INDOLE + SERINE ON GROWTH & ALKALOID PRODUCTION

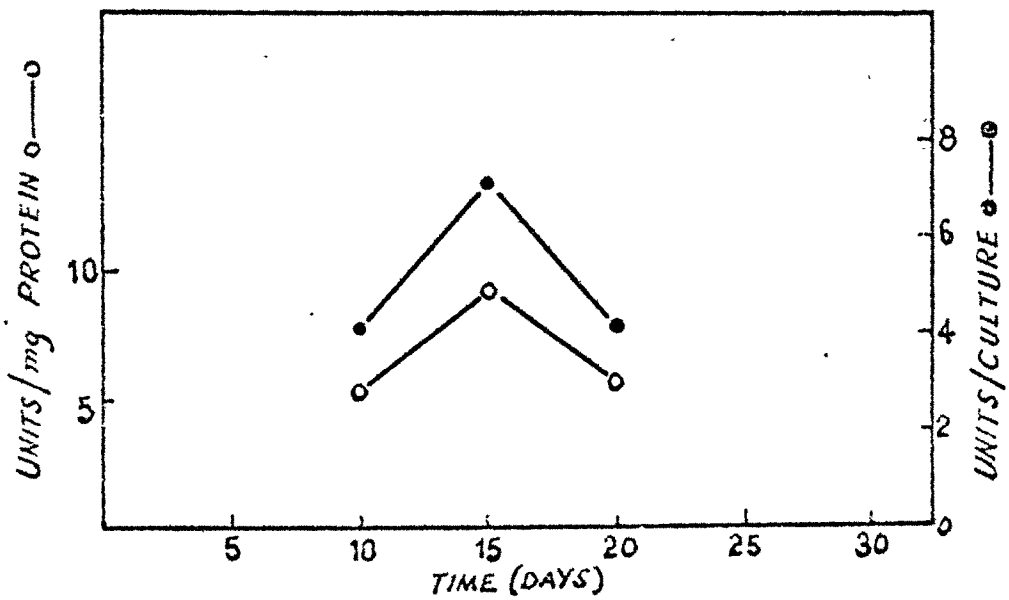

FIG. 64 - EFFECT OF INDOLE + SERINE ON TRYPTOPHAN SYNTHETASE ACTIVITY

SECTION - J

Studies on Amino acids during the course of culture in Evolvulus suspension cultures.

Cultures grown in MS medium containing 2% sucrose and supplemented with 2.0 mg/l 2,4-D and 0.4 mg/l kinetin were analysed for their amino acid contents. Tryptophan, Serine and Methionine which are directly connected with alkaloid production were studied quantitatively. The remaining amino acids were qualitatively analysed during the course of culture. The tissue was harvested at the intervals of 5 days for periodical determination of amino acids.

In all seven amino acids were found to be present (Table 41) during the course of culture. Among the amino acids studied qualitatively, Aspargine and Glutamine were observed to be present during the entire course of culture from day 5 till day 30. Cysteine was detected only on day 20 and 25 of culture; whereas Phenyl-alanine was detected on all days of culture except on day 5.

The quantitative estimations of Tryptophan, Serine and Methionine are presented in Table 42 and Fig. 65. Tryptophan was found to be present in comparatively less amounts on days 5, 25 and 30 and was maximum on day 20 of culture. Serine increased steadily from day 5 to day 15 when its highest value was attained. It remained absent from day 20 to 25. Methionine was relatively less on days 5 and 30; but reached its maximum value on day 15. It was not detected on days 20 and 25.

Table - 41. Amino acids in E. alsinoides suspension cultures during the course of cultures.

Inoculum : 200±20 mg tissue by fresh weight (dry weight 11.08 mg) in 25 ml of MS medium containing 2% sucrose and supplemented with 2 mg/l 2,4-D and 0.4 mg/l kinetin.

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

Amino acids	Period of culture					
	5	10	15	20	25	30
Aspargine	+	+	+	+	+	+
Serine	+	+	+	-	-	+
Tryptophan	+	+	+	+	+	+
Methionine	+	+	+	-	-	+
Cysteine	-	-	-	+	+	-
Glutamine	+	+	+	+	+	+
Phenylalanine	-	+	+	+	+	+

+ = Present - = Absent

Table - 42. Periodic study of Tryptophan, Serine and Methionine quantitatively in E. alsinoides suspension cultures.

Amino acids	µgms/100 mg dry weight of tissue					
	5	10	15	20	25	30
Tryptophan	300	900	1500	3000	600	250
Serine	1000	2000	3500	-	-	250
Methionine	200	500	850	-	-	150

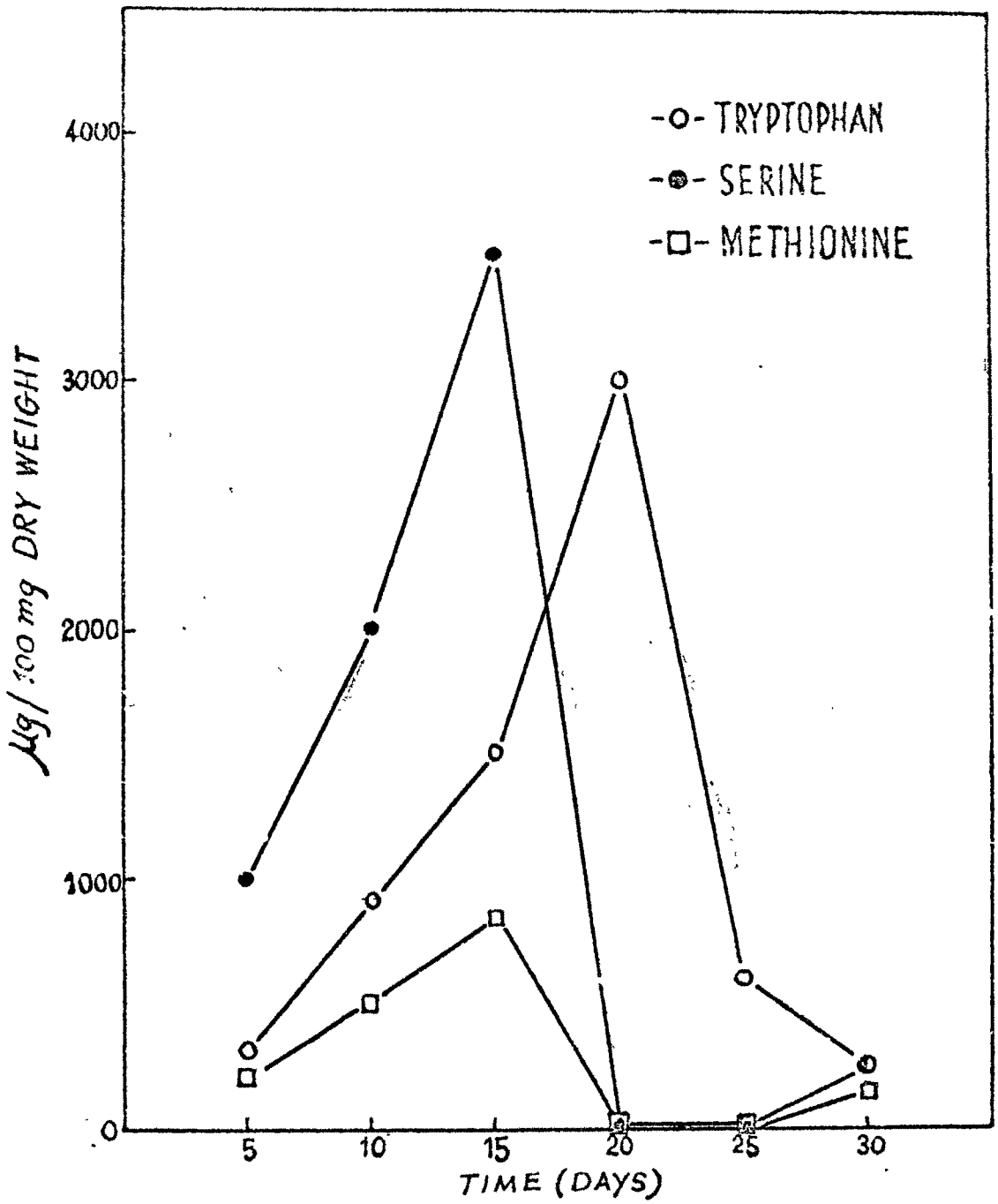

FIG. 65 - PERIODIC STUDY OF AMINO ACIDS.

SECTION - K

Phosphate inhibition of alkaloid and its control by tryptophan and its analogues in Evolvulus suspension culture.

Inorganic phosphate at higher concentrations has been shown to inhibit ergot alkaloid production in fungi. Further, addition of L-tryptophan to phosphate inhibited cultures had profound effect in restoring the alkaloid production; whereas homologues of Tryptophan and Mevalonic acid could not restore the production of alkaloids inhibited by Inorganic phosphate. Similar studies were, therefore, carried out in Evolvulus alsinoides also to examine their effects in cultures of higher plants. The experiments carried out in this direction are explained in the following subsections.

K - 1 : Phosphate inhibition of alkaloid in Evolvulus suspension cultures.

Inorganic phosphate was incorporated in the medium in the form of Potassium dihydrogen orthophosphate (KH_2PO_4) at 340 mg/l, 680 mg/l and 1020 mg/l. The normal cultures contained 170 mg/l of inorganic phosphate.

Measured aliquots of cell suspension weighing 200±20 mg tissue by fresh weight were transferred to 25 ml of MS medium

(supplemented with 2.0 mg/l 2,4-D and 0.4 mg/l kinetin in addition to 2% sucrose) containing 340 mg, 680 mg or 1020 mg/l of KH_2PO_4. The culture vessels were incubated at 25±2°C, in continuous light for a period of 30 days. A fixed number of replicates were harvested at the interval of 5 days for periodic measurement of growth and alkaloid production.

The results presented in Table 43 and Fig. 66 showed that at the lowest level of inorganic phosphate tested, the growth of tissue had a marked promotory effect, registering about 30 fold increase in fresh weight and 19 fold increase in dry weight. Further, a remarkable feature in the case of cultures containing higher amounts of inorganic phosphate was that the growth of tissue did not cease at day 25 but continued to increase till day 30. At 680 mg/l level of inorganic phosphate (4 times higher than the normal dose), the tissue did not show appreciable increase in growth compared to the cultures in 340 mg/l inorganic phosphate level (Table 44, Fig. 68). At the highest level of phosphate tested (1020 mg/l) there was no appreciable change in growth of tissue except a slight increase in fresh weight and dry weight.

The alkaloid production was not much effected by doubling the level of inorganic phosphate in the medium. The initial inoculum contained 0.009%; while at 340 mg/l of phosphate also the quantity of alkaloid present remained almost the same (Table 43, Fig. 66). However, the alkaloid production was

Table - 43. Periodic changes in growth, alkaloid production and tryptophan synthetase activity in E. alsinoides suspension cultures at high inorganic phosphate levels.

Inoculum : 200±20 mg tissue by fresh weight (dry weight 12.07 mg) in 25 ml of MS medium containing 2% sucrose and supplemented with 2.0 mg/l 2,4-D and 0.4 mg/l kinetin and 340 mg/l Inorganic phosphate.

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

Days	Fresh weight (mg/cult.)	Dry weight (mg/cult.	Alkaloid (%)	Alkaloid (µg/cult.)	Tryptophan Synthetase (units/mg protein)	Tryptophan Synthetase (units/cult.)
0	200.00 (±1.368)	12.07 (±0.231)	0.009	-	-	-
5	497.7 (±1.447)	27.44 (±0.134)	0.006	1.64	-	-
10	1156.6 (±2.392)	56.7 (±0.574)	0.006	3.4	8.620	28.9
15	1404.3 (±3.527)	61.2 (±0.669)	0.007	4.28	19.230	105.3
20	4389.5 (±24.466)	167.2 (±1.501)	0.009	16.04	12.5	164.587
25	5373.1 (±31.645)	193.2 (±1.603)	0.009	17.38	10.0	161.190
30	5907.9 (±45.764)	208.6 (±1.855)	0.007	14.6	-	-

Data represents average of five replicates.

Figures in parenthesis represent standard error.

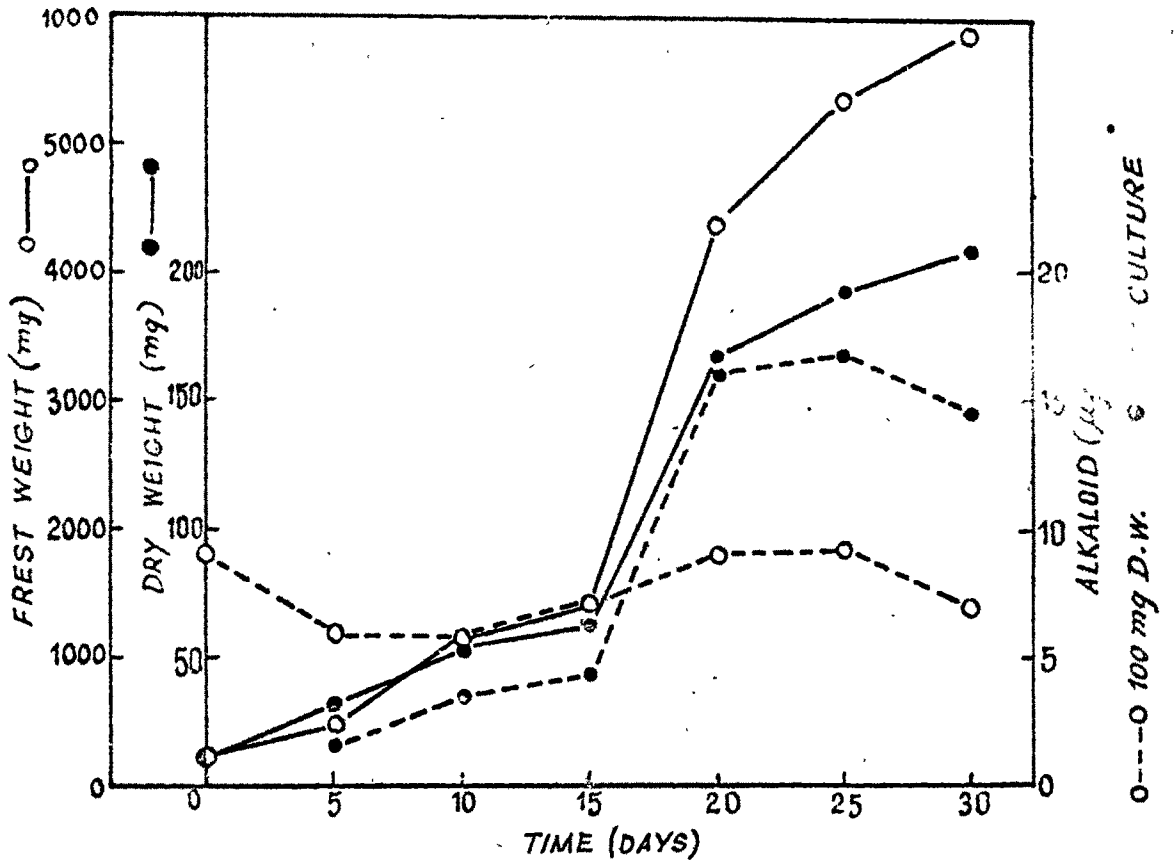

FIG.66-EFFECT OF 340 mg/l INORGANIC PHOSPHATE ON GROWTH & ALKALOID PROD.

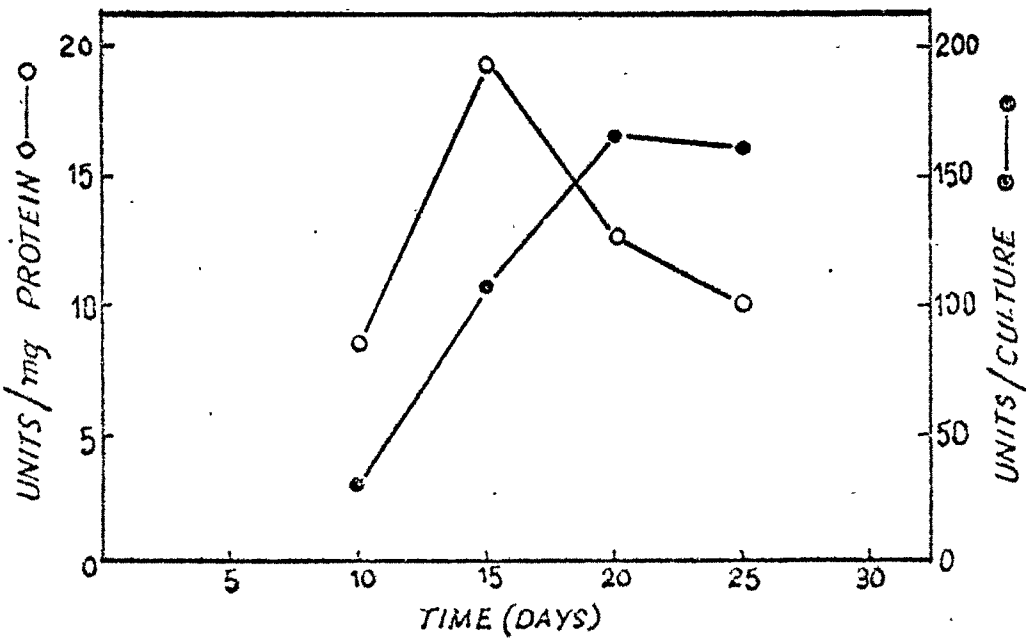

FIG. 67-EFFECT OF 340 mg/l INORGANIC PHOSPHATE ON TRYPTOPHAN SYNTHETASE ACT.

Table - 44. Periodic changes in growth, alkaloid production and tryptophan synthetase activity in E. alsinoides suspension cultures at high inorganic phosphate levels.

Inoculum : 200±20 mg tissue by fresh weight (dry weight 12.07 mg) in 25 ml of MS medium containing 2% sucrose and supplemented with 2.0 mg/l 2,4-D and 0.4 mg/l kinetin and 680 mg/l inorganic phosphate.

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

Days	Fresh weight (mg/cult.)	Dry weight (mg/cult.)	Alkaloid		Tryptophan Synthetase	
			(%)	(µg/cult.)	(units/mg protein)	(units/cult.)
0	200.00 (±1.368)	12.07 (±0.231)	0.009	-	-	-
5	551.8 (±0.215)	31.6 (±0.107)	0.006	1.89	-	-
10	1123.6 (±2.258)	56.24 (±0.565)	0.007	3.94	11.803	42.212
15	1878.6 (±6.313)	93.7 (±0.675)	0.007	6.55	21.875	164.325
20	2779.4 (±13.819)	118.06 (±1.249)	0.007	8.26	5.970	69.475
25	3894.1 (±27.126)	177.6 (±1.126)	0.007	12.43	5.273	120.920
30	5846.6 (±24.341)	201.8 (±1.977)	0.007	14.12	-	-

Data represents average of five replicates.

Figures in parenthesis represent standard error.

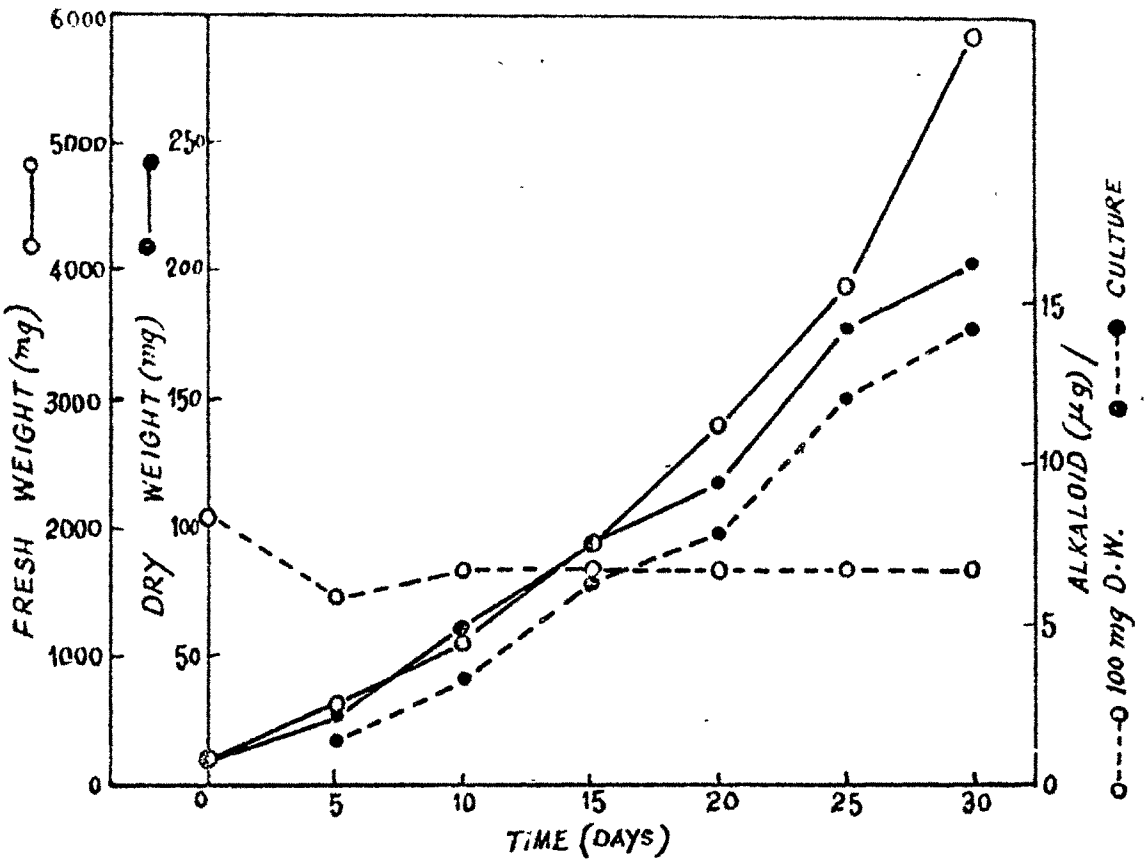

FIG. 68-EFFECT OF 680 mg/l INORGANIC PHOSPHATE ON GROWTH & ALKALOID PROD.

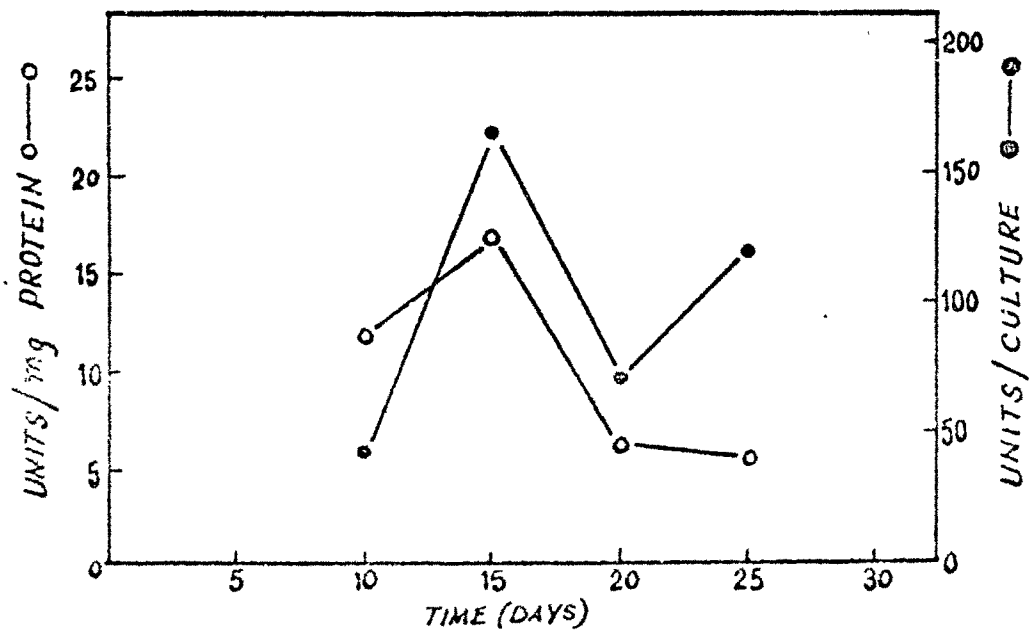

FIG. 69-EFFECT OF 680mg/l INORGANIC PHOSPHATE ON TRYPTOPHAN SYNTHETASE ACTIVITY

reduced, the highest value registered being 0.007% in presence of higher (680 mg/l) level of phosphate (Table 44, Fig. 68). At the highest level of phosphate (1020 mg/l), alkaloid production was further inhibited showing 0.006% on day 25 (Table 45, Fig. 70).

Assay of tryptophan synthetase was carried out in the above treatments by harvesting a fixed number of replicates at the intervals of 5 days till 30 days of culture. The specific activity of tryptophan synthetase expressed per milligram protein for callus tissue over a period of 30 days at 340 mg/l inorganic phosphate is shown in Table 43 and Fig. 67. Enzyme activity was not detected till day 10, after which it increased. After recording the peak value on day 15, the activity gradually declined until no activity was registered on day 30.

The specific activity of tryptophan synthetase expressed per milligram protein and units/culture in presence of 680 mg/l inorganic phosphate level in the medium is shown in Table 44 and Fig. 69. The activity was absent on day 5. After attaining the peak value on day 15, the activity declined to low values on days 20 and 25 and was nil on day 30.

Tryptophan synthetase activity could not be detected in tissues growing on media containing 1020 mg/l inorganic phosphate during the entire culture period (Table 45).

Table - 45. Periodic changes in growth, alkaloid production and tryptophan synthetase activity in *E. alsinoides* suspension cultures at high inorganic phosphate levels.

Inoculum : 200±20 mg tissue by fresh weight (dry weight 12.07 mg) in 25 ml of MS medium containing 2% sucrose and supplemented with 2.0 mg/l 2,4-D and 0.4 mg/l kinetin and 1020 mg/l inorganic phosphate.

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

Days	Fresh weight (mg/cult.)	Dry weight (mg/cult.)	Alkaloid		Tryptophan Synthetase	
			(%)	(µg/cult.)	(units/mg protein)	(units/cult.)
0	200.00 (±1.368)	12.07 (±0.231)	0.009	-	-	-
5	506.2 (±0.759)	28.8 (±0.432)	0.006	1.73	-	-
10	1023.8 (±1.187)	51.1 (±0.591)	0.006	3.06	-	-
15	2658.3 (±3.069)	121.5 (±0.141)	0.006	7.29	-	-
20	3251.9 (±3.755)	133.8 (±0.155)	0.006	8.02	-	-
25	6049.3 (±4.984)	210.05 (±0.121)	0.006	12.6	-	-
30	6043.7 (±4.839)	210.01 (±0.178)	0.006	12.6	-	-

Data represents average of five replicates.

Figures in parenthesis represent standard error.

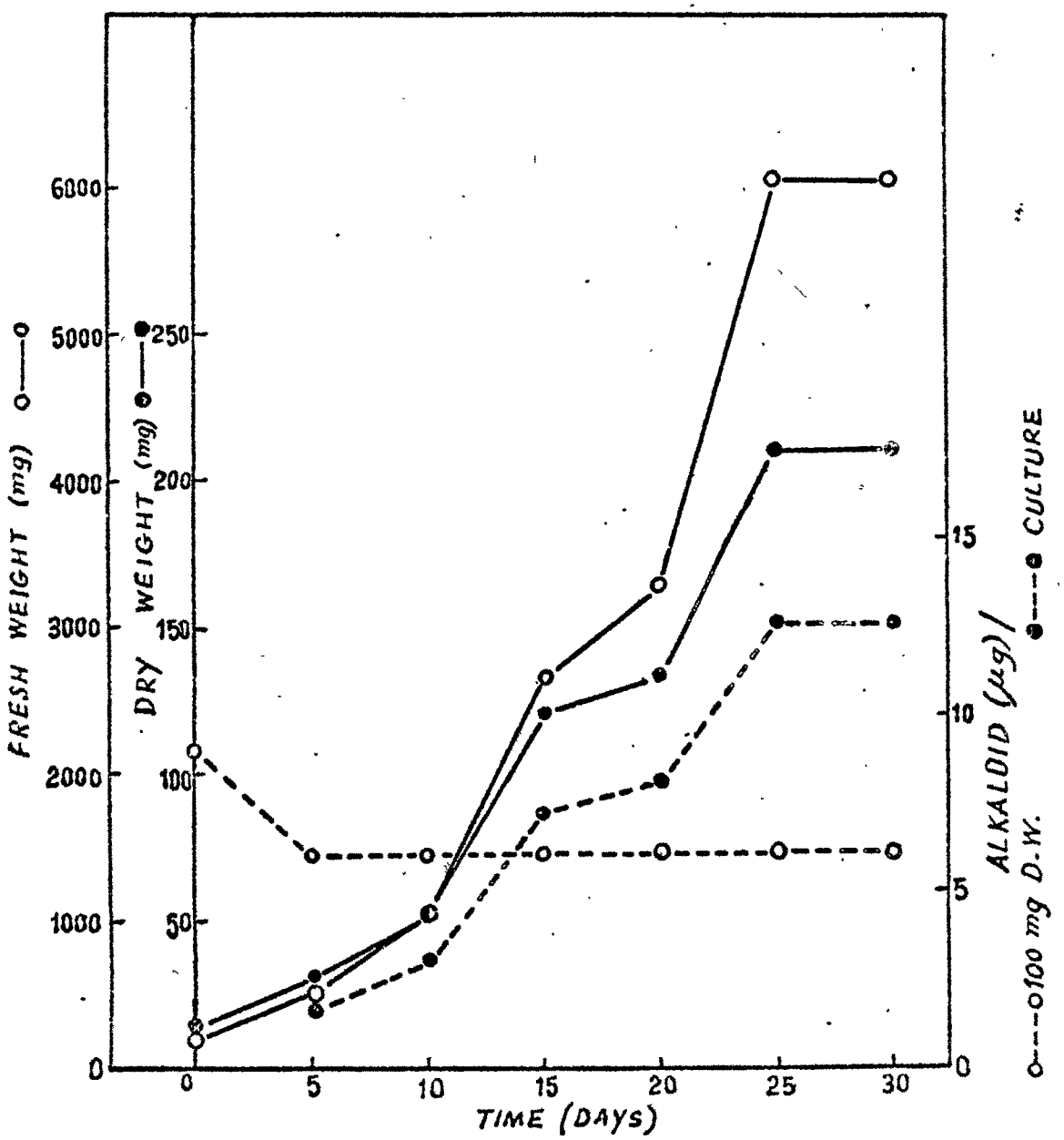

FIG. 70-EFFECT OF 1020 mg/ℓ INORGANIC PHOSPHATE ON GROWTH & ALKALOID PRODUCTION

K - 2 : Effect of L-tryptophan on phosphate inhibited alkaloid production

Measured aliquots of cell suspension weighing 200±20 mg tissue by fresh weight was transferred to 25 ml of MS medium (supplemented with 2.0 mg/l 2,4-D and 0.4 mg/l kinetin in addition to 2% sucrose) containing 1020 mg/l inorganic phosphate and 5 mM L-tryptophan. The culture vessels were incubated at 25±2°C in continuous light for a period of 30 days. A fixed number of replicates was harvested at the interval of every 5 days for periodic determination of growth and alkaloid production.

The growth of tissue was considerably inhibited by the addition of L-tryptophan. The fresh weight increase was about 9 fold and dry weight increase was about 6 fold. But the alkaloid production was restored back as in normal (control) cultures by the addition of L-tryptophan as shown in Table 46 and Fig. 71.

In tissues growing in media containing the highest level of inorganic phosphate tested, the addition of L-tryptophan had no stimulating effect on tryptophan synthetase activity. The tryptophan synthetase activity remained inhibited even after the addition of L-tryptophan (Table 46).

Table - 46. Effect of L-tryptophan on Inorganic phosphate inhibition of alkaloid in *E. alsinoides* suspension cultures.

Inoculum : 200±20 mg tissue by fresh weight (dry weight 11.01 mg) in 25 ml of MS medium containing 2% sucrose and supplemented with 2.0 mg/l 2,4-D and 0.4 mg/l kinetin and 1020 mg/l Inorganic phosphate + 5 mM, L-tryptophan.

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

Days	Fresh weight (mg/cult.)	Dry weight (mg/cult.)	Alkaloid (%)	Alkaloid (µg/cult.)	Tryptophan Synthetase (units/mg protein)	Tryptophan Synthetase (units/cult.)
0	200.00 (±1.134)	11.01 (±0.185)	0.009	-	-	-
5	650.9 (±0.321)	53.8 (±0.167)	0.006	3.2	-	-
10	1212.8 (±1.412)	57.6 (±0.521)	0.007	3.7	-	-
15	1532.12 (±3.161)	63.72 (±0.671)	0.009	5.7	-	-
20	1723.13 (±5.832)	67.12 (±0.167)	0.01	6.7	-	-
25	1765.37 (±3.841)	67.02 (±0.521)	0.011	7.4	-	-
30	1743.21 (±7.164)	66.91 (±1.232)	0.011	7.4	-	-

Data represents average of five replicates.

Figures in parenthesis represent standard error.

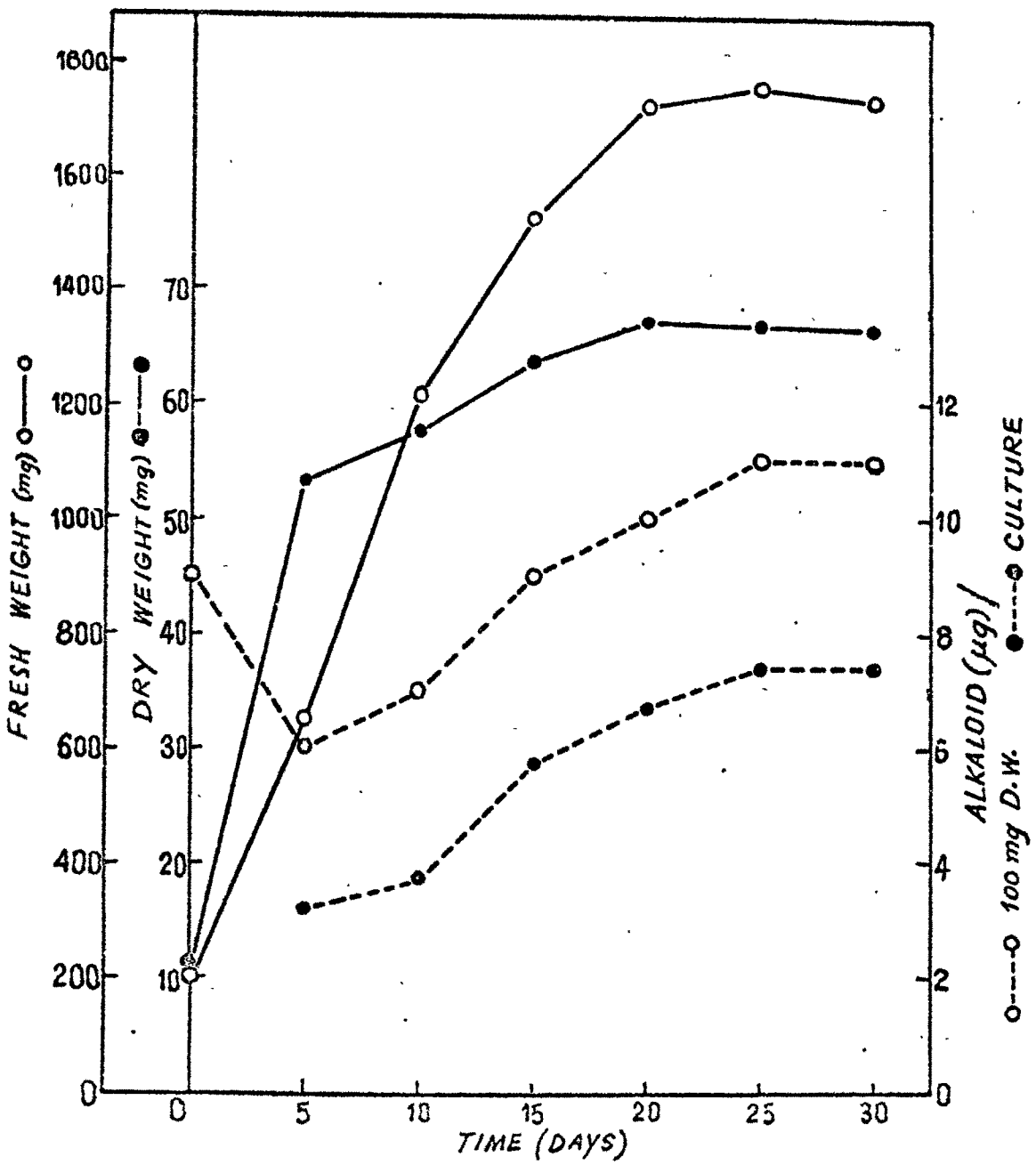

FIG.71- CONTROL OF PHOSPHATE INHIBITION OF ALKALOID PRODUCTION BY L-TRYPTOPHAN.

K - 3 : Effect of Mevalonic acid on phosphate inhibited alkaloid production.

Measured aliquots of cell suspension weighing 200±20 mg tissue by fresh weight was transferred to 25 ml of MS medium (supplemented with 2.0 mg/l 2,4-D and 0.4 mg/l kinetin in addition to 2% sucrose) containing 1020 mg/l inorganic phosphate and 5 mM, Mevalonic acid. The culture vessels were incubated at 25±2°C in continuous light for a period of 30 days. A fixed number of replicates were harvested at the interval of every 5 days for periodic estimation of growth and alkaloid production.

The growth of tissue was promoted by the addition of Mevalonic acid. The fresh weight increase was about 30.5 fold and dry weight increase was about 20 fold. On the other hand, Mevalonic acid did not have any promotory effect on alkaloid production, i.e., it could not reverse the inhibition by high levels of inorganic phosphate (Table 47 and Fig. 72).

Similar to earlier treatment (L-tryptophan + 1020 mg/l inorganic phosphate), Mevalonic acid also could not restore the tryptophan synthetase activity, inhibited by high levels of inorganic phosphate (Table 47).

K - 4 : Effect of 5-methyl tryptophan on phosphate inhibited alkaloid production .

5-methyl tryptophan at 1 mM concentration was incorporated

Table - 47. Effect of Mevalonic acid on Inorganic phosphate inhibition of alkaloid in *E. alsinoides* suspension cultures.

Inoculum : 200±20 mg tissue by fresh weight (dry weight 11.01 mg) in 25 ml of MS medium containing 2% sucrose and supplemented with 2.0 mg/l 2,4-D and 0.4 mg/l kinetin and 1020 mg/l Inorganic phosphate + 5 mM, Mevalonic acid.

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

Days	Fresh weight (mg/cult.)	Dry weight (mg/cult.)	Alkaloid		Tryptophan Synthetase	
			(%)	(µg/cult.)	(units/mg protein)	(units/cult.)
0	200.00 (±1.134)	11.01 (±0.185)	0.009	-	-	-
5	510.12 (±0.812)	29.07 (±0.432)	0.006	1.7	-	-
10	1025.7 (±1.134)	52.01 (±0.521)	0.006	3.1	-	-
15	2751.02 (±6.123)	120.72 (±1.132)	0.006	7.2	-	-
20	3450.8 (±10.016)	135.6 (±1.321)	0.006	8.1	-	-
25	6129.3 (±17.032)	210.8 (±2.361)	0.006	13.0	-	-
30	6126.7 (±15.232)	210.67 (±3.126)	0.006	12.6	-	-

Data represents average of five replicates.

Figures in parenthesis represent standard error.

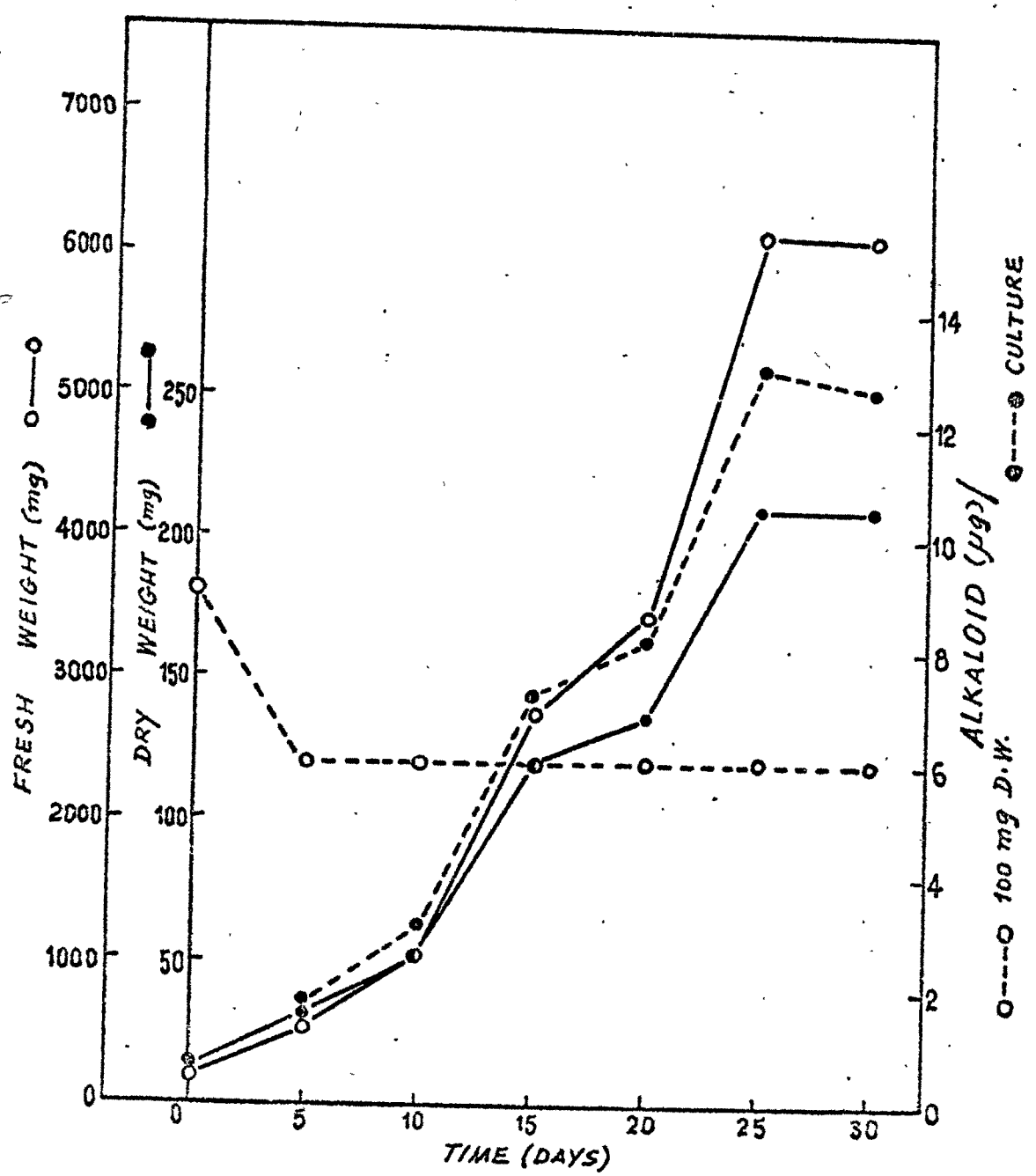

FIG. 72 - CONTROL OF PHOSPHATE INHIBITION OF ALKALOID PRODUCTION BY MEVALONIC ACID.

in the medium containing high levels of inorganic phosphate to find out if Methyl tryptophan could overcome the high phosphate inhibition of alkaloid production.

The growth of tissue was inhibited by Methyl tryptophan, recording a fresh weight increase of only about 5 fold and dry weight increase of 4 fold. Further, Methyl tryptophan also could not reverse the alkaloid inhibition by high levels of inorganic phosphate (Table 48, Fig. 73).

As in the case of L-tryptophan and Mevalonic acid, 5-methyl tryptophan also could not restore the tryptophan synthetase activity, inhibited by high levels of inorganic phosphate (Table 48).

Table - 48. Effect of 5-methyl tryptophan on Inorganic phosphate inhibition of alkaloid production in E. alsinoides suspension cultures.

Inoculum : 200±20 mg tissue by fresh weight (dry weight 11.01 mg) in 25 ml of MS medium containing 2% sucrose and supplemented with 2.0 mg/l 2,4-D and 0.4 mg/l kinetin and 1020 mg/l Inorganic phosphate + 2 mM, 5-Methyl tryptophan.

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

Days	Fresh weight (mg/cult.)	Dry weight (mg/cult.)	Alkaloid		Tryptophan Synthetase	
			(%)	(µg/cult.)	(units/mg protein)	(units/cult.)
0	200.00 (±1.134)	11.01 (±0.185)	0.009	-	-	-
5	452.16 (±1.267)	25.161 (±0.876)	0.006	1.51	-	-
10	512.231 (±1.861)	28.231 (±1.101)	0.006	1.69	-	-
15	585.216 (±2.012)	28.701 (±1.081)	0.006	1.7	-	-
20	685.217 (±2.721)	34.165 (±1.761)	0.006	2.04	-	-
25	698.23 (±2.812)	35.21 (±1.251)	0.006	2.1	-	-
30	697.165 (±3.102)	35.01 (±1.012)	0.006	2.1	-	-

Data represents average of five replicates.

Figures in parenthesis represent standard error.

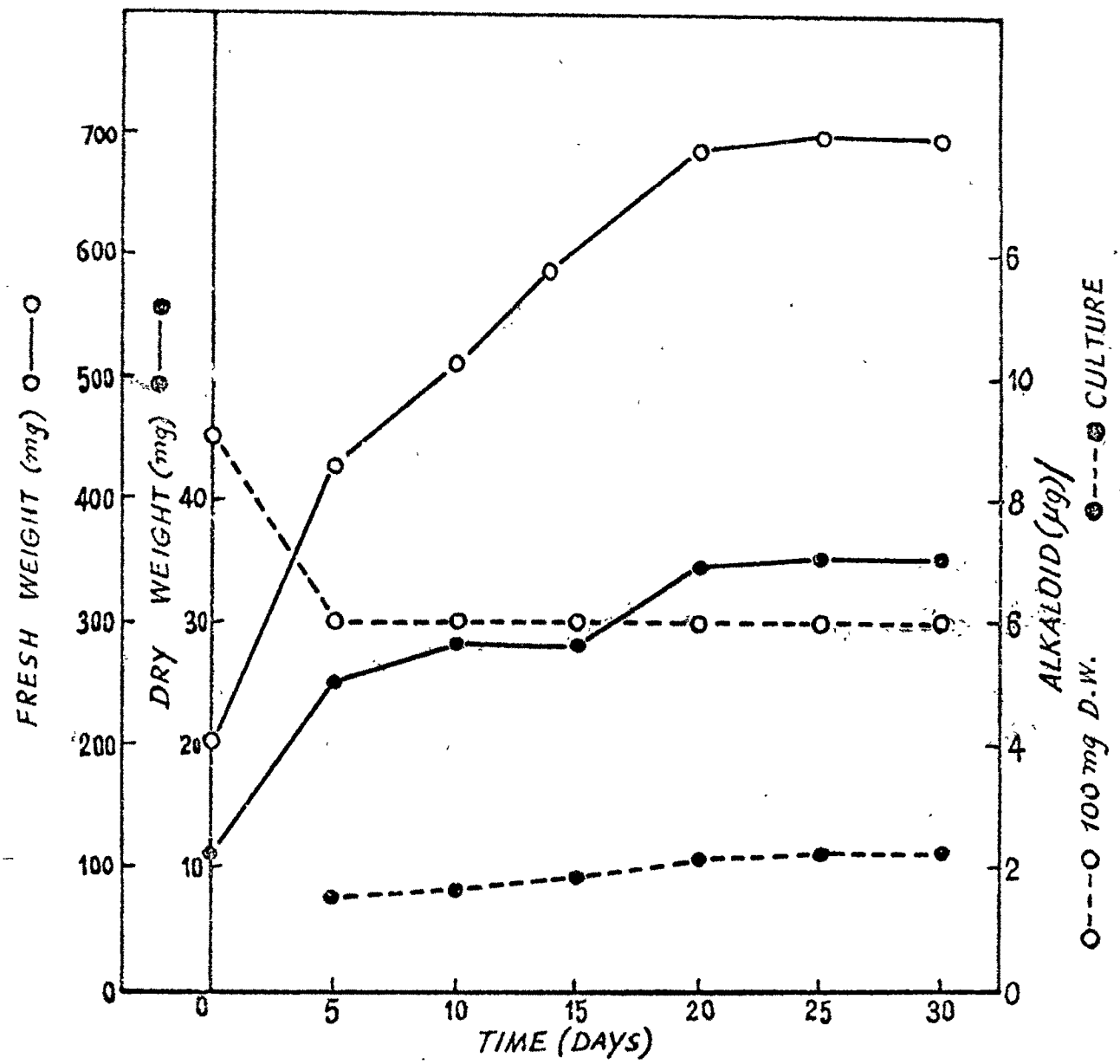

FIG.73-EFFECT OF 5-METHYL TRYPTOPHAN ON INORGANIC PHOSPHATE INHIBITION OF ALKALOID PRODUCTION.

SECTION - L

Influence of co-factors of DMAT synthetase on growth and alkaloid production in Evolvulus suspension cultures.

Dimethylallyl pyrophosphate : L-tryptophan dimethylallyl transferase (DMAT synthetase), the first enzyme in the pathway of ergot alkaloids catalysing the formation of 4-(γ, γ - dimethylallyl)- tryptophan (DMAT) from Dimethylallyl pyrophosphate and L-tryptophan could not be studied since Radio Tracer facility was not available. However, the influence of Fe^{2+}, Mg^{2+} and Ca^{2+} which are known to activate the DMAT synthetase was examined on total alkaloid biosynthesis. The control medium contained Fe^{2+}, Mg^{2+} and Ca^{2+} at the concentrations of 0.1 mM, 1.5 mM and 3 mM respectively. Measured aliquots of cell suspension weighing 200±20 mg tissue by fresh weight were transferred to 25 ml of MS medium (supplemented with 2.0 mg/l 2,4-D and 0.4 mg/l kinetin in addition to 2% sucrose) containing 6 mM or 10 mM of Ca^{2+}, 3 mM or 6 mM of Mg^{2+} and 1 mM or 4 mM of Fe^{2+} to examine the effect of these co-factors separately on alkaloid production. The culture vessels were incubated at 25±2°C in continuous light for 25 days; after which they were harvested to determine growth and alkaloid production. The results are presented in Table 49 and Fig. 74.

Cultures containing higher levels of Ca^{2+}(6 mM and 10 mM)

had a promotory effect on alkaloid production. 10 mM Ca^{2+} stimulated the highest production of alkaloid. The growth of tissue by fresh weight and dry weight, however, was suppressed at the higher level of Ca^{2+} tested (Table 49 and Fig. 74).

Mg^{2+} at 3 mM concentration did not have any marked effect on growth of tissue; but alkaloid synthesis was increased considerably. On the other hand, 6 mM concentration of Mg^{2+} had an inhibitory effect on growth of tissue, without any influence on the alkaloid production (Table 49 and Fig. 74).

Fe^{2+} at 1 mM concentration did not have any appreciable effect on growth of tissue; but the alkaloid production was considerably enhanced. However, the higher concentration of Fe^{2+} (4 mM) inhibited the growth of tissue as well as alkaloid production (Table 49, Fig. 74).

Table - 49. Effect of different levels of calcium, magnesium and iron on growth and alkaloid production in E. alsinoides suspension cultures.

Inoculum : 200±20 mg tissue by fresh weight (dry weight 11.06 mg) in 25 ml of MS medium containing 2% sucrose and supplemented with 2.0 mg/l 2,4-D and 0.4 mg/l kinetin and

(A) Control - (Fe, 0.1 mM, Ca, 3 mM, Mg, 1.5 mM)

(B) Ca^{++} - 6 mM (C) Ca^{++} - 10 mM

(D) Mg^{++} - 3 mM (E) Mg^{++} - 6 mM

(F) Fe^{++} - 1 mM (G) Fe^{++} - 4 mM

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

Treatment	Fresh weight (mg/cult.)	Dry weight (mg/cult.)	Alkaloid (%)	Alkaloid (µg/cult.)
A	3450.02 (± 9.632)	149.01 (±2.123)	0.01	14.9
B	3482.1 (±11.654)	149.87 (±3.813)	0.014	21.0
C	3001.2 (± 9.164)	128.71 (±2.187)	0.016	20.6
D	3461.15 (±10.056)	148.9 (±3.643)	0.013	19.0
E	2172.04 (± 7.1418)	118.1 (±3.154)	0.01	12.0
F	3325.16 (±13.167)	141.7 (±1.012)	0.013	18.0
G	1501.01 (± 5.081)	95.72 (±0.785)	0.008	7.6

Data represents average of five replicates.

Figures in parenthesis represent standard error.

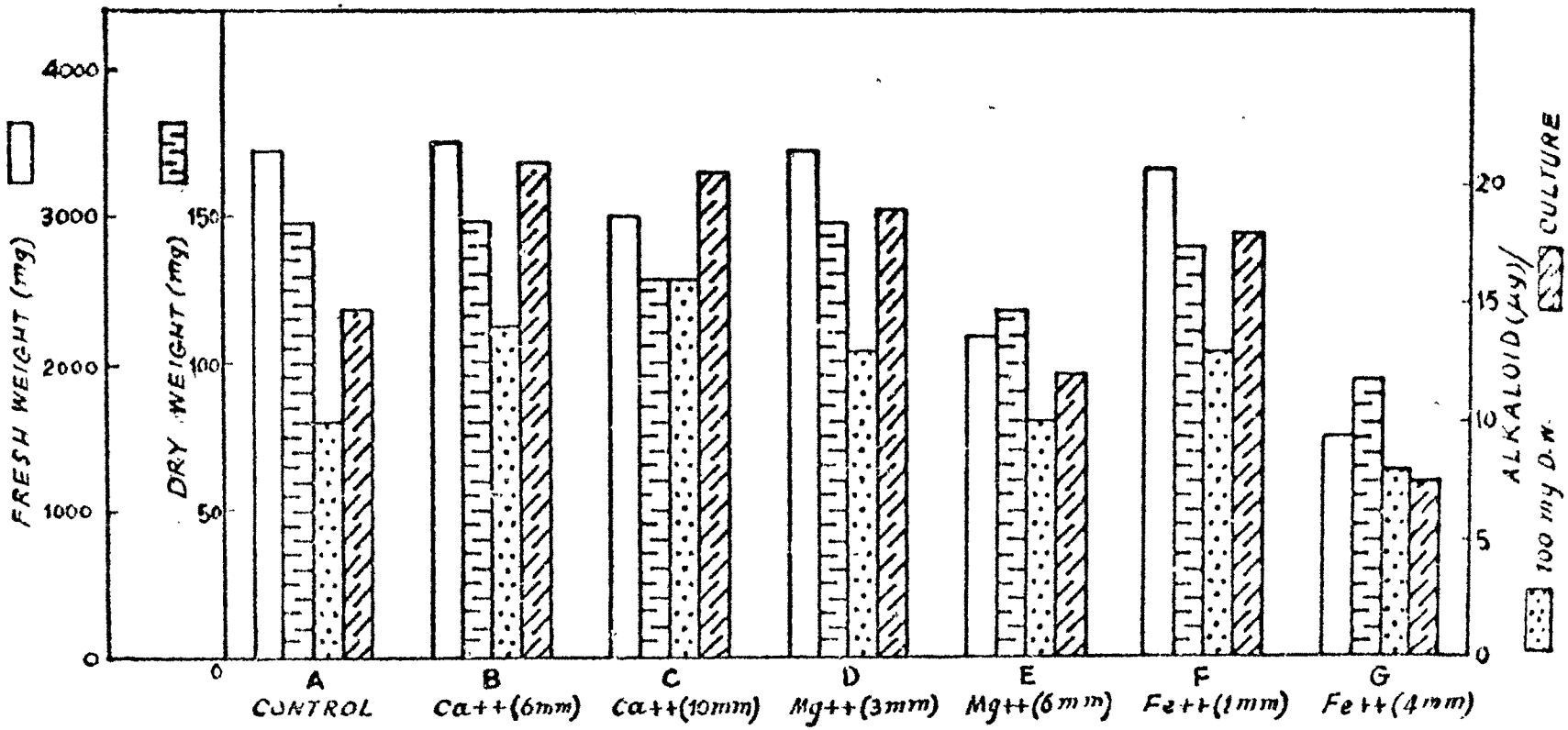

FIG.74 - INFLUENCE OF COFACTORS & INHIBITORS OF DMAT SYNTHETASE ON GROWTH & ALKALOID PRODUCTION

SECTION - M

Changes in growth and alkaloid production with passages in culture of Evolvulus cell suspensions

It has been observed that the cells loose their morphogenetic potentialities as they age in cultures. Growth rates of tissue as well as production of secondary metabolites are also reported to decline with time.

In the present studies, the cultures initiated in early 1978 have undergone 24 transfers to freshly prepared media at regular intervals of about 30 days. To check if any changes have occurred in growth and alkaloid patterns of the tissues, the tissues were assayed after every 4 subcultures for growth and alkaloid determinations. The data is presented in Table 50 and Fig. 75.

The growth of tissue did not show any considerable change in fresh and dry weights after 1st and 4th subcultures. Distinct enhancement in growth was noticed at 8th and 12th subcultures. Growth values showed slight decline at the 16th transfer. There was, however, appreciable reduction in growth after 20th and 24th subcultures.

Similar pattern was observed with alkaloid production also. There was no change in alkaloid contents in tissues

Table - 50. Changes in growth and alkaloid production in serial subcultures of E. alsinoides suspension cultures.

Inoculum : 200±20 mg tissue by fresh weight (dry weight 11.05 mg) in 25 ml of MS medium containing 2% sucrose and supplemented with 2.0 mg/l 2,4-D and 0.4 mg/l kinetin.

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

subculture	Fresh weight (mg/cult.)	Dry weight (mg/cult.)	Alkaloid (%)	Alkaloid (µg/cult.)
1st	4092.6 (±14.761)	198.8 (±3.528)	0.012	23.8
4th	4006.24 (±13.265)	197.02 (±3.801)	0.012	23.6
8th	4214.62 (±11.164)	205.02 (±3.236)	0.015	30.7
12th	4201.15 (±12.362)	204.78 (±2.187)	0.015	30.7
16th	4001.1 (±10.865)	196.12 (±2.321)	0.012	23.5
20th	3500.01 (±11.021)	150.73 (±3.001)	0.01	15.0
24th	3450.02 (±9.632)	149.01 (±2.123)	0.01	14.9

Data represents average of five replicates.

Figures in parenthesis represent standard error.

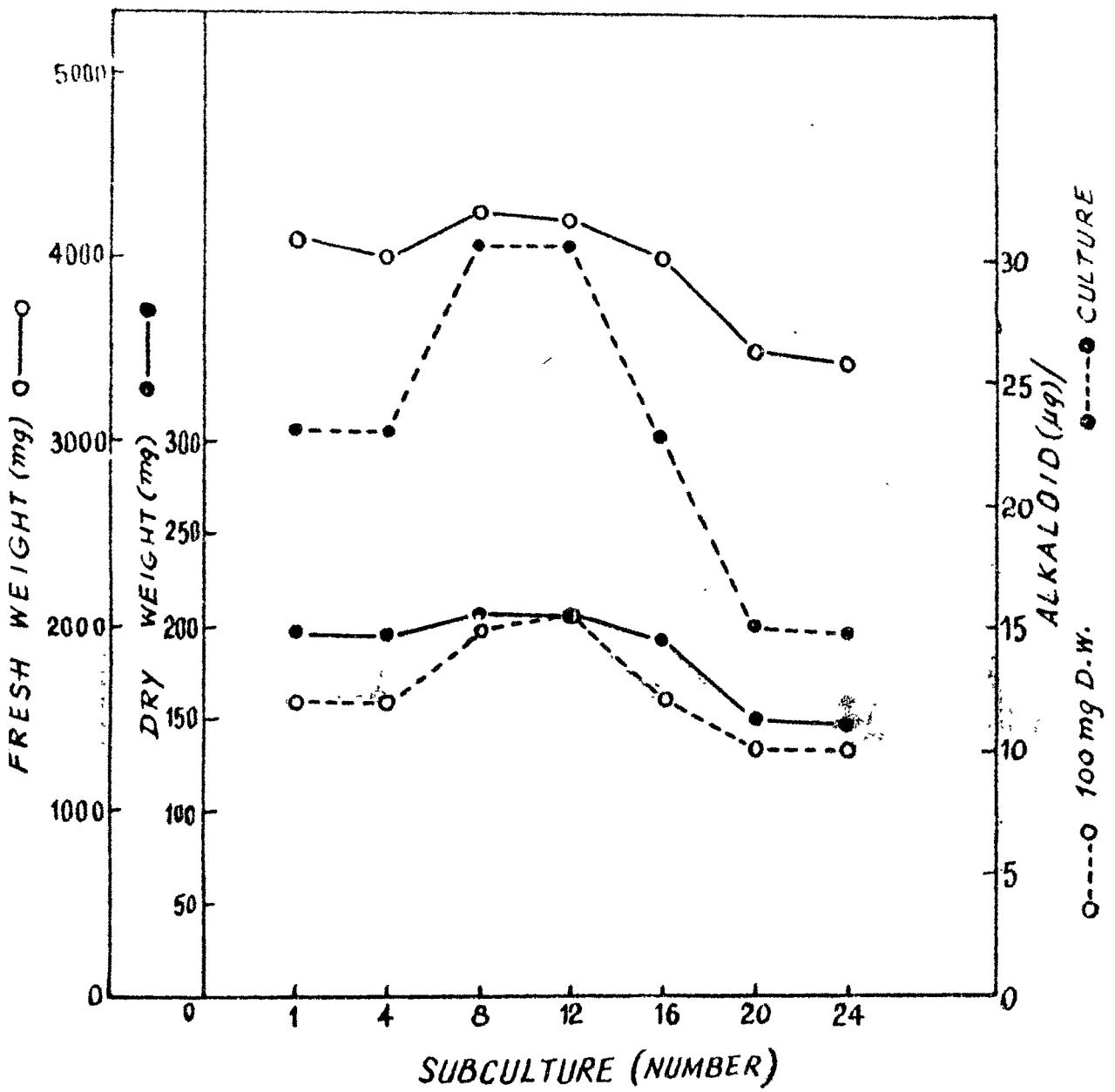

FIG.75-CHANGE IN GROWTH & ALKALOID PRODUCTION WITH PASSAGES IN CULTURE.

after 1st and 4th subcultures. This was followed, as in the case of growth, with increased alkaloid production recorded after 8th and 12th transfers, both percentage-wise and on yield per culture basis. After a slight reduction in 16th subculture, the alkaloid content registered decline at 20th and 24th transfers.

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