RESULTS

RESULTS

(A) *IN VIVO* SCREENING OF PLANTS

Plants of *Withania somnifera* (L.) Dunal were collected from different localities and screened for the presence of alkaloids. The alkaloid was isolated, identified and quantified as per the standard procedures. Among the alkaloids, tropine alkaloid was identified as the authentic sample of this was available. A superior 'elite' plant was identified based on the highest tropine contents. Further, individual organs of this plant were screened for their tropine contents so that the site of synthesis / accumulation could be confirmed.

Experiment 1 : Screening of *Withania somnifera* (L.) Dunal plants for their alkaloid contents

The objective of this experiment was to detect the presence of alkaloid tropine in the plant and to quantify it.

W. somnifera plants were collected in their vegetative and fertile stages of development from M S University Campus (Fig. 1a, b). They were washed under tap water, rinsed in distilled water, dried at room temperature and powdered. These samples were extracted for their alkaloids following the procedures described by Roberts and James (1947) which have been given in Chapter II Materials and Methods. A Herbarium sheet of the plant was prepared, identified and maintained in the Departmental Herbarium.

Fig. 1 (a) A plant of *Withania somnifera* from M S University Campus

(b) A fertile twig

a

b

Fig·1

Silica gel plated TLC plates were prepared and spotted with the chloroform soluble extract. They were dried in oven and sprayed with Dragendorff's reagent, when orange coloured spots were developed. This test showed the presence of alkaloid extracted in chloroform soluble fraction. Further, these spots were eluted, dried, redissolved in chloroform and once again used for TLC along with the authentic sample of tropine. It was observed that the R_f value of isolated compounds was 0.35 which coincided with the values of the authentic sample (Fig. 2). This confirmed that tropine alkaloid was extracted in the chloroform soluble fraction of the extract.

Results recorded in Table III indicate that vegetative plant of *W. somnifera* accumulated on dry weight basis 0.10% of the alkaloid which was enhanced to 0.43% as the plant reached flowering stage. ~~to fertile stage of development~~.

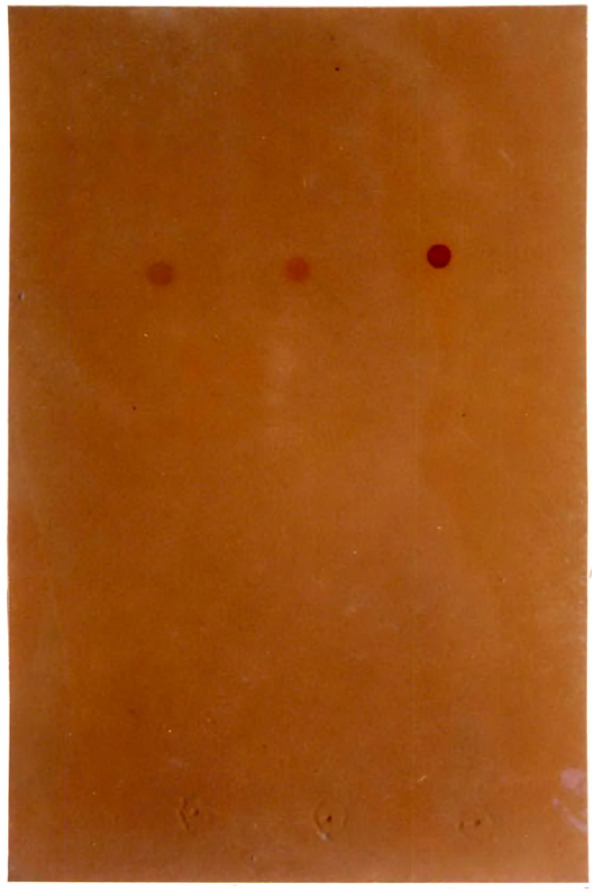
The results of this experiment clearly confirmed that tropine alkaloid was synthesized and accumulated by this plant.

Experiment 2 : Selection of 'elite' *W. somnifera* (L.) Dunal plant

W. somnifera plants were collected in their vegetative and flowering ~~fertile~~ stages of development from (a) Ellora Park (b) M S University Campus, (c) Karelibag, (d) Chhani and (e) Harni localities. These plants were processed for isolation of their tropine contents.

Observations recorded in Table IV indicate that highest quantity of the alkaloid was accumulated plants growing in the Ellora Park area when they were in fertile stage of development (0.55%). M S University Campus plants followed next, as their alkaloid content was 0.43% when they were in

Fig. 2 TLC of extracts of *W. somnifera* (a,b) along with authentic sample of tropine (c)



a b c

Fig·2

Not clear!
A line drawing of ... would have sufficed
situation.

Table III: Alkaloid contents in *Withania somnifera* (L.) Dunal plants from M S University Campus

Sr. No.	Stage of development of plant	% alkaloid content
1.	Vegetative	0.10
2.	Fertile	0.43

% dry weight basis

Table IV: Alkaloid profile of *W. somnifera* (L.) Dunal plants from various localities

Sr. No.	Locality	% alkaloid content in plants	
		Vegetative	Fertile
1.	Ellora park	0.21	0.55
2.	M S University Campus	0.16	0.43
3.	Karelibag	0.10	0.35
4.	Chhani	0.05	0.10
5.	Harni	0.03	0.08

% dry weight basis

their fertile stage of development. Plants growing in other localities did contain tropine alkaloid but there was further reduction in their quantities. Plants growing in Harni area recorded lowest alkaloid contents.

Results of this experiment showed that plant gorowing in Ellora Park area synthesized/accumulated highest alkaloid contents amongst the plants screened and hence they were identified as 'elite' superior plants.

Experiment 3 : Screening of individual organs of 'elite' plant

In this experiment,the individual organs of an 'elite' plant raised from the seeds under controlled environmental conditions were used. The procedure employed for screening was that of Roberts and James (1947) as given in Chapter II 'Materials and Methods.'

Results recorded in Table IV clearly indicate that roots of this plant accumulate 0.25% and stem 0.10% of tropine alkaloid, when calculated on dry weight basis. The leaves of this plant contained 0.13%, while fruits accumulated 0.08% of the alkaloid.

From this result it was obvious that roots were the sites of accumulation of this alkaloid.

Experiment 4 : Alkaloid contents of roots of *W.somnifera* from various localities

As the roots indicated the site of accumulation of tropine alkaloid, roots from four to five year old plant growing in various localities (a) Ellora Park (b) M S University Campus (c) Karelibag (d) Chhani and (e) Harni were

Table V: Tropine profile of individual organs of *W. somnifera* 'elite' plant

Sr. No.	Plant Organ	% alkaloid
1	Roots	0.25
2	Stem	0.10
3	Leaves	0.13
4	Fruits	0.08

% dry weight basis

collected. Out of the five samples of roots, plants growing in Ellora Park and M S University Campus had roots which were hard and woody (Fig. 3a, b). Roots of the plants collected from Karelibag and Chhani were thin (Fig. 3c, d). Plants of Harni area possessed short and stunted roots with profuse laterals (Fig. 3e). These roots were processed in the usual manner and screened for their alkaloid contents.

Results recorded in Table VI indicate that there is variation in the quantities of the alkaloids of these root samples of plants from various localities, the highest being in the roots of plants growing in Ellora Park area, followed by M S University Campus plants. In plants growing in Harni area the roots recorded minimum alkaloid contents (0.03%).

This experiment clearly indicates that the roots of the plants of the same age collected from various localities differed in their alkaloid contents.

(B) *IN VITRO* EXCISED ROOT CULTURE

In this section experiments were conducted for the establishment of axenic cultures of roots, since roots were proved to be the site of the synthesis/accumulation of tropine alkaloid (Section A).

The nutritional/hormonal requirements supporting active and continuous growth of cultured roots were standardised. The growth parameters studied were, length of main axis, number of laterals produced from it, and biomass production in terms of fresh and dry weights during culture period of four to eight weeks. The tropine alkaloid profile was investigated during

Fig. 3 Root samples of *W. somnifera* plants collected from various localities

(a) Ellora Park,

(b) M S University Campus,

(c) Karelibag,

(d) Chhani and

(e) Harni

a

b

c

Fig. 3

d

e

Fig. 3

Table 10: Tropine content in root samples of plants growing in different localities

Sr. No.	Locality	% tropine
a	Ellora Park	0.25
b	M S University Campus	0.20
c	Karelibag	0.10
d	Chhani	0.05
e	Harni	0.03

% dry weight basis

the experimental period and it was expressed on percentage dry weight basis. Further experimental work was conducted to bring about the increase in the alkaloid contents in cultured roots.

Experiment 5 : Selection of suitable culture medium for excised roots

The composition of culture medium particularly the macro, micro salts and vitamins exert profound effect on the growth of cultured roots. In the present experiment, two known media (40 ml) White (1954) (Table I) and Murashige and Skoog (1962) (Table II) were inoculated with root tips (1-2 cm) excised from aseptically germinated seedlings. Culture flasks were maintained on gyratory shaker (120 rpm) in culture room (25 ± 2° C) in 16 h photoperiod (1000 lux).

Results recorded in Table VII showed that the main axis of the root reached to 2.9 cm in length in White's medium without producing a single lateral root at the end of four weeks culture period (Fig. 4a). On the other hand, in M S medium, although the growth of main axis was poor (1.5 cm), but it produced 1.3 lateral roots (Fig. 4b).

This shows that the M S medium supported the growth of excised roots in culture better when compared with the White's medium. As M S medium was found to be superior to White's medium, it was employed in further experimental work.

Table VII : Effect of White / Murashige and Skoog's medium on the growth of excised roots in culture

Medium : White / MS medium + sucrose (2%)

Inoculum : 1-2 cm roots with tips

Incubation : 4 weeks in culture room at 25 ± 2° C in 16 h photo period (1000 lux).

Sr. No.	Medium	Length of main axis cm	Number of laterals
1	White's	2.9 ± 1.3	-
2	MS	1.5 ±0.3	1.3 ± 0.43

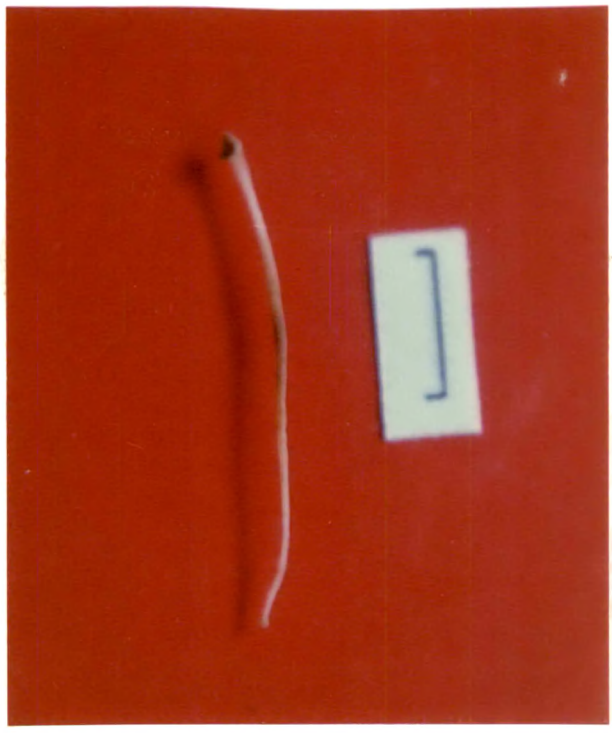
Mean of six replicates with standard deviation

- No response

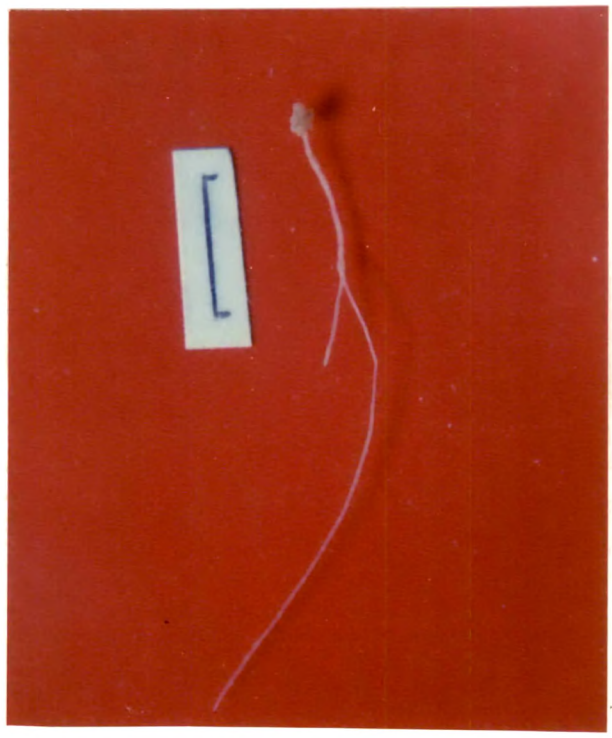
Fig. 4 Cultured roots after four weeks in

(a) White medium

(b) Murashige and Skoog medium



a



b

Fig · 4

Experiment 6 : Standardisation of MS medium salt concentrations for optimal growth of excised roots

This experiment was conducted to find out the optimal dose of macro and micro salts, and vitamins as present in MS medium which could support maximum growth of cultured roots.

Excised roots (1-2 cm) with their tips intact were inoculated in MS (40 ml) medium containing standard doses of macro and micro salts, and vitamins; in the second set, they were reduced to one half strength and in the third set, their concentrations were increased to double strength of the original medium. These media were supplemented with sucrose (2%). Cultural conditions were maintained constant as given in Chapter II, Materials and Methods.

Results recorded in Table VIII showed that the roots cultured in one half strength MS medium grew well and ~~reached to~~ attained 3.1 cm length of the main axis with production of 3.3 lateral roots (Fig.5), ~~While~~ whereas the excised roots cultured in MS medium, with standard doses of salts ~~reached~~ grew to 1.5 cm in length of the main axis with 1.3 lateral root production. Further, doubling the concentration of macro and micro salts and vitamins of the MS medium it was observed that the length of the main axis declined (1.0 cm), without any production of lateral roots.

Thus, it was confirmed from these results that the macro and micro salts, and vitamins present in MS medium in their half strength supported optimal growth of cultured roots.

Table VIII: Effect of MS medium salts at various concentrations (Half / Standard / Double) on excised root culture

Medium : MS (Half / Standard / Double) + sucrose (2%)

Inoculum (Explant) : 1-2 cm root with tip

Incubation : 4 weeks in culture room at 25 ± 2° C in 16 h photoperiod (1000 lux)

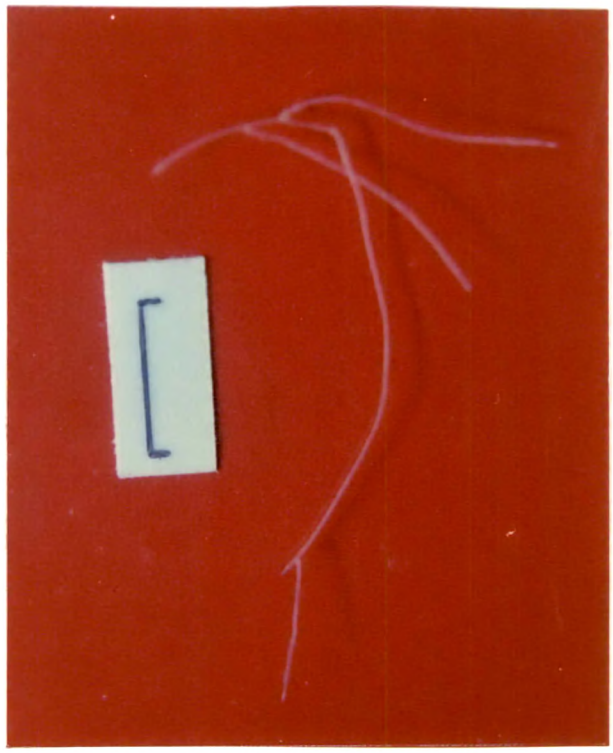
Sr. No.	Medium concentration	Length of main axis cm	No. of laterals
1	Half	3.1 ± 0.06	3.3 ± 0.43
2	Standard	1.5 ± 0.3	1.3 ± 0.43
3	Double	1.0 ± 0	-

Mean of six replicates with standard deviation

- No response

Fig. 5 Roots grown in M S medium (half strength) + sucrose (2%)

After 4 weeks of culture period



Fig· 5

Experiment 7 : Effect of phytohormones on the growth of excised roots in culture

(i) Effect of cytokinins Kn/BAP

Excised roots cultured *in vitro* require exogenous supply of phytohormones for their growth and development during the culture period. This experiment was undertaken to find out the requirements of cytokinins *viz.*, Kn, BAP to the roots for their growth.

Healthy roots with tips (1-2 cm) excised from aseptically germinated seeds were inoculated in standard MS medium (40 ml) containing sucrose (2%) and supplemented with Kn/BAP 0.5-6 µM/l individually.

Results indicated that Kn or BAP when tested individually at various concentations (0.5-6 µM/l) showed no improvement in the growth of the cultured roots either in the length of main axis or in the production of laterals.

Hence, it was clear that no exogenous supply of cytokinins was necessary for the growth of these cultured roots.

(ii) Effect of auxins IAA/IBA/NAA/2,4-D

Effect of each auxin IAA/IBA/NAA/2,4-D on the growth of excised cultured roots of *W. somnifera* was investigated by incorporating them at various concentrations (0.5-6 µM/l) individually in MS medium (40 ml) containing sucrose (2%). Experimental procedures followed were as given in Chapter II 'Materials and Methods'.

Results recorded in Table IX show that excised roots cultured in IAA (0.5 µM/l) containing medium supported the growth of main axis to 2.5 cm with 7.3 lateral root production. The fresh and dry weights recorded were 7.2 mg and 0.21 mg respectively. It was observed that increase in IAA level to 2 µM/l produced highest increase in the length of main axis of root (3.1 cm) along with highest number of lateral root production (12.6). At this level of IAA, biomass values were 12.4 mg and 0.37 mg in terms of fresh and dry weights respectively. Further, increase in IAA level in the medium resulted in reduction of all growth parameters. Hence, IAA at 2 µM/l level could be taken as optimal level for continuous growth of cultured roots.

Studies on the application of IBA at various levels, indicated that upto 2 µM/l level there was a linear increase in all the growth parameters studied viz., 2.5 cm length of main axis, 15 number of laterals, 14.8 mg fresh weight and 0.44 mg dry weight. Further increase in IBA level of the culture medium to 4 and 6 µM/l brought decline in these growth parameters. Hence, 2 µM/l of IBA was suitable for excised roots of this plant. Comparing the growth parameters, in presence of IAA the length of main axis was highest. Roots cultured in IBA containing medium supported the number of lateral root production and their fresh and dry weights also were increased as seen in results of Table IX.

However, cultured roots treated with NAA (0.5-6 µM/l) showed no enhancement in the length of the root main axis and production of lateral roots.

Table IX : Effect of auxins (IAA / IBA / NAA / 2,4-D) on the growth of excised roots in culture

Medium : MS root culture medium + surcose (2%) + IAA / IBA / NAA / 2, 4-D (0.5 - 6 µM/ l)

Inoculum : 1 -2 cm root with tip .

Incubation : 4 weeks in culture room at 25 ± 2° C in 16 h photo period (1000 lux)

Sr. No.	Auxin (µm/l)	Length of main root axis (cm)	No. of lateral roots produced	Fresh wt. (mg)	Dry wt. (mg)
	IAA				
1	0.5	2.5 ± 0.06	7.3 ± 0.43	7.2 ± 0.2	0.21 ± 0.003
2	1.0	2.8 ± 0.06	9.6 ± 0.46	9.6 ± 0.16	0.28 ± 0.04
3	2.0	3.1 ± 0.1	12.6 ± 1.8	12.4 ± 0.9	0.37 ± 0.006
4	4.0	2.5 ± 0.06	8 ± 0.43	8.1 ± 0.13	0.23 ± 0.006
5	6.0	2.1 ± 0.06	5 ± 1.8	5.3 ± 0.13	0.16 ± 0.04
	IBA				
6	0.5	2.1 ± 0.06	10 ± 1.8	10.4 ± 0.3	0.31 ± 0.01
7	1.0	2.1 ± 0.06	12 ± 0.46	11.8 ± 0.26	0.35 ± 0.006
8	2.0	2.5 ± 0.006	15 ± 0.06	14.8 ± 0.13	0.44 ± 0.006
9	4.0	2.3 ± 0.06	7 ± 0.43	6.6 ± 0.26	0.19 ± 0.006
10	6.0	2 ± 0	2 ± 0.43	2.1 ± 0.06	0.06 ± 0
	NAA				
11	0.5	2 ± 0	-	-	-
12	1.0	2 ± 0	-	-	-
13	2.0	2 ± 0	-	-	-
14	4.0	2 ± 0	-	-	-
15	6.0	2 ± 0	-	-	-
	2,4-D				
16	0.5	C	-	-	-
17	1.0	C	-	-	-
18	2.0	C	-	-	-
19	4.0	-	-	-	-
20	6.0	-	-	-	-

Mean of six replicates with standard deviation

- No response

C - Callusing

Incorporating 2,4-D at various levels in culture medium induced callus at the root tips. The time required for callus induction decreased with the increasing levels of 2,4-D in the culture medium.

Thus, IAA/IBA each at 2 μM/l level was found suitable for continuous growth of cultured root. In fact IAA favoured optimal growth of main axis of root, while IBA favoured highest number of lateral root formation as well as supported the production of biomass in terms of fresh and dry weights (Table IX).

Experiment 8 : Effect of IAA in combination with IBA on the growth of excised roots in culture

Results of previous experiment proved that the application of IAA and IBA at 2 μM/l each, was beneficial for the cultured roots as the length of main axis, production of laterals as well as the biomass values were considerably increased. Hence, this experiment was conducted to find out their synergestic effect keeping the levels of IAA and IBA at 0.5 to 6 μM/l each. The growth parameters studied were length of main axis of roots and the number of lateral roots produced.

Results recorded in Table X showed that IAA in combination with IBA from 0.5 to 2 μM/l each, produced linear increase in the length of main root axis upto 4.1 cm with 30 lateral root production. The laterals were initiated within one week (Fig. 6a) and their growth was slow initially but in the third and fourth week it was very fast. It was noted that as the length of lateral roots increased, the growth of the main axis slowed down. The length

Table 14 : Effect of IAA and IBA in combination on the growth of cultured roots

Medium : MS root culture medium + sucrose (2%) + IAA (0.5 -6 µM/l) + IBA (0.5-6 µM/l)

Inoculum : Explant 1 - 2 cm roots with tips .

Incubation : 4 weeks in culture room at 25 ± 2°C in 16h photoperiod (1000 lux)

Sr. No.	Auxin µ M/l	Length of main axis cm					Number of laterals produced				
	IBA	0.5	1	2	4	6	0.5	1	2	4	6
	IAA										
1	0.5	2.3 ± 0.1	2.2 ± 0.06	2 ± 0	2 ± 0	2 ± 0	10.3 ± 1.1	12.6 ± 1.8	15.6 ± 1.4	7.3 ± 0.46	6 ± 0.8
2	1	2.5 ± 0.06	3.3 ± 0.06	3 ± 0.06	2.5 ± 0.06	2.1 ± 0.03	11.6 ± 1.1	18 ± 0.6	21 ± 0.6	15.6 ± 0.46	10.3 ± 0.43
3	2	2.5 ± 0.06	3.5 ± 0.06	4.1 ± 0.06	3 ± 0	2.8 ± 0.03	15 ± 0	23.3 ± 0.4	30 ± 0.6	23 ± 0.6	17.3 ± 0.43
4	4	2 ± 0	2.2 ± 0.06	2.8 ± 0.06	2.5 ± 0	2 ± 0	5.3 ± 0.43	8.6 ± 0.86	18.3 ± 1.1	13.6 ± 0.86	11.3 ± 0.43
5	6	2 ± 0	2 ± 0	2 ± 0	2 ± 0	2.06 ± 0.04	2 ± 0	5.3 ± 0.43	11.3 ± 1.1	6.6 ± 0.46	2.3 ± 0.43

Mean of six replicates with standard deviation

Fig. 6 Roots grown in

'root culture medium' + sucrose (2%) + IAA (2 μM/l) +

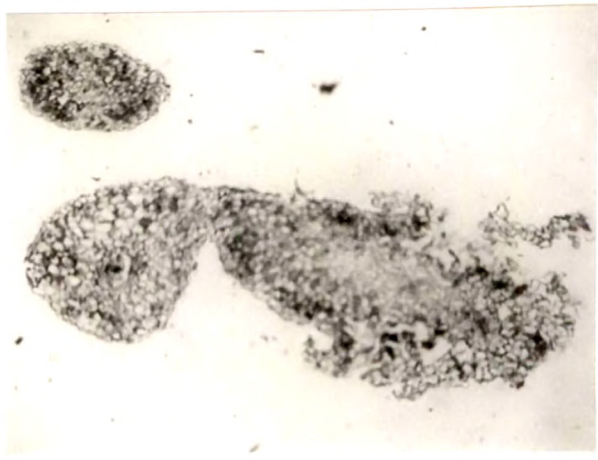
IBA (2 μM/l)

(a) After one week showing numerous root primordia

(b) T.S of root with a primordium (160 X)

(c) After four weeks

a



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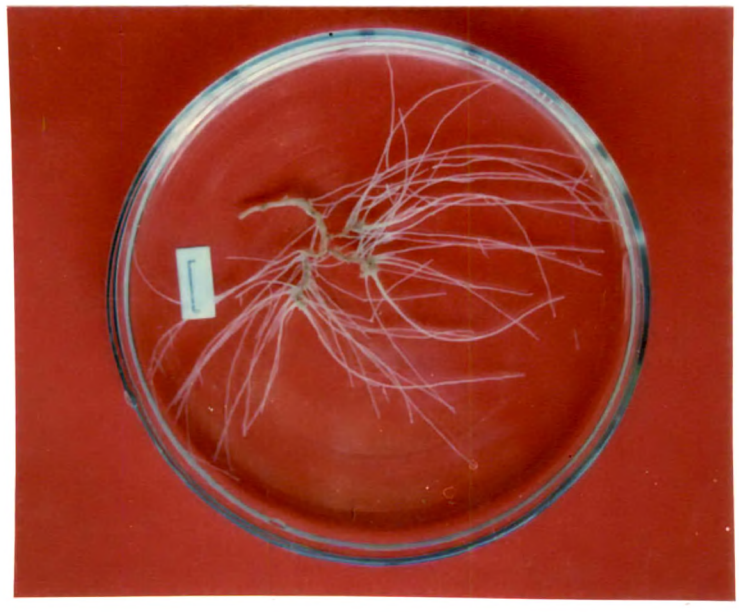


c

Fig · 6

of lateral roots was usually three times the length of main axis. Besides, a few lateral roots became thicker and showed the formation of secondary laterals (Fig. 6c). With the increase in IAA and IBA levels to 4 μM/l and 6 μM/l each, the values of these growth parameters declined.

Histological studies during root primordium induction showed that the epidermis burst open and root primordium pierced out from the underlying tissues (Fig. 6b).

Thus, IAA in combination with IBA at 2 μM/l each, caused the synergistic ~~combination resulting in~~ beneficial effects on the growth of excised roots in culture. Hence, MS medium containing sucrose 2% and IAA with IBA at 2 μM/l each was selected as the 'root culture medium' for *W. somnifera*.

Experiment 9 : Effect of different carbohydrates (Glucose / Sucrose / Mannitol) on the growth of excised roots in culture

The excised cultured roots require an exogenous energy source in the medium, and hence the root culture medium (40 ml) was incorporated with glucose / sucrose / mannitol at 2% and their biomass production after four weeks was recorded.

Results recorded in Table ~~XI~~ 15 indicate that after four week culture period, the roots in glucose supplemented medium recorded fresh weight 838 ± 9 mg and dry weight 35 ± 2 mg (Fig. 7a). At the same concentration of sucrose, the biomass values were 2018 ± 20 mg fresh weight and 95 ± 3 mg dry weight (Fig. 7b), ~~While~~ whereas in mannitol, the cultured roots recorded very little

Table XI : Effect of different carbohydrates (Glucose / Sucrose / Mannitol) on growth of cultured roots

Medium : MS root culture medium + Glucose / Sucrose / Mannitol (2%) + IAA + IBA (2 µM/l, each)

Inoculum : 10 roots with tips (1-2 cm)
Fresh weight - 10 ± 0.6 mg.
Dry Weight - 0.4 ± 0.02 mg.

Incubation : 4 weeks in culture room at 25 ± 2° C for 16 h photoperiod (1000 lux)

Sr. No.	Carbohydrate	Fr. wt. mg	Dry wt mg
1	Glucose	838 ± 9	35 ± 2
2	Sucrose	2018 ± 20	95 ± 3
3	Mannitol	276 ± 3	15 ± 1

Mean of six replicates with standard deviation.

Fig. 7 Cultured roots in

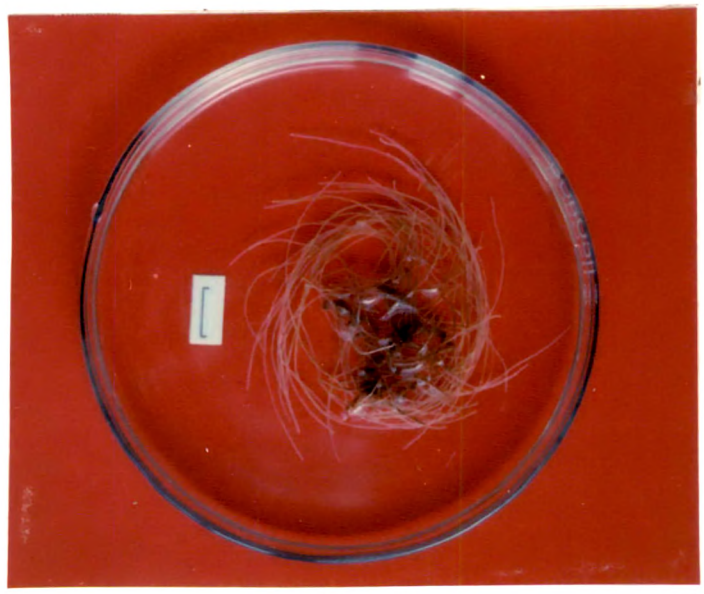
'root culture medium' + IAA (2 μM/l) + IBA (2 μM/l)

(a) + Glucose (2%)

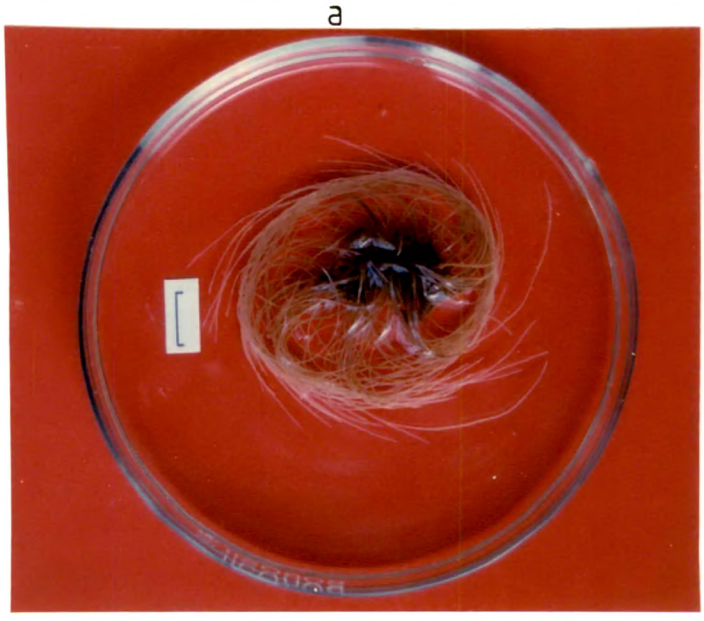
(b) + Sucrose (2%)

(c) + Mannitol (2%)

After four weeks of culture period



a



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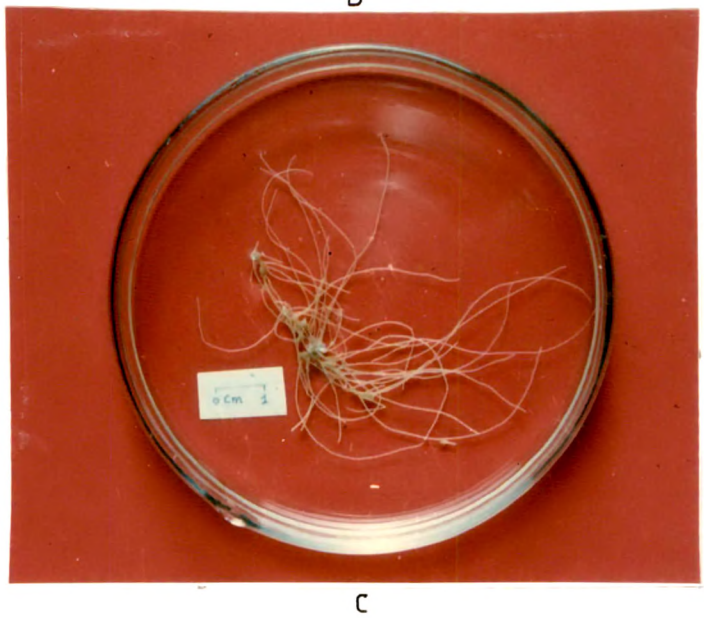


c

Fig. 7

biomass production in terms of fresh and dry weights ~~which were~~ namely, 276 ± 3 mg and 15 ± 1 mg respectively (Fig. 7c).

This experiment proved that sucrose, the disaccharide supplied requisite dose of energy to the cultured roots and thereby the growth parameters recorded were the highest when compared with glucose and mannitol.

Experiment 10 : Determination of optimal level of sucrose necessary for continuous growth of cultured roots

The root culture medium (40 ml) was supplemented with sucrose at various levels (0-4 %). Results recorded after four weeks of culture period, showed that the cultured roots turned black in the absence of sucrose. Incorporation of sucrose at 1% improved the growth of excised roots as the biomass production recorded in terms of fresh and dry weights were 1337 ± 13 mg and 65 ± 5 mg respectively (Fig. 8). These parameters reached their highest values when sucrose concentration in the culture medium was 2%. At this level of sucrose the fresh and dry weights were 2012 ± 20 mg and 95 ± 7 mg respectively. With further increase in sucrose level to 3%, the biomass values recorded were 1750 ± 18 mg and 82 ± 6 mg respectively which showed reduction in their amounts. Still further reduction in the growth parameters were recorded at 4% of sucrose as seen in ~~the results~~ (Fig. 8).

Thus, sucrose at 2% was proved to be the optimal level necessary for the best normal growth of cultured roots.

Fig. 8 Growth of excised roots at various concentrations of sucrose after four weeks

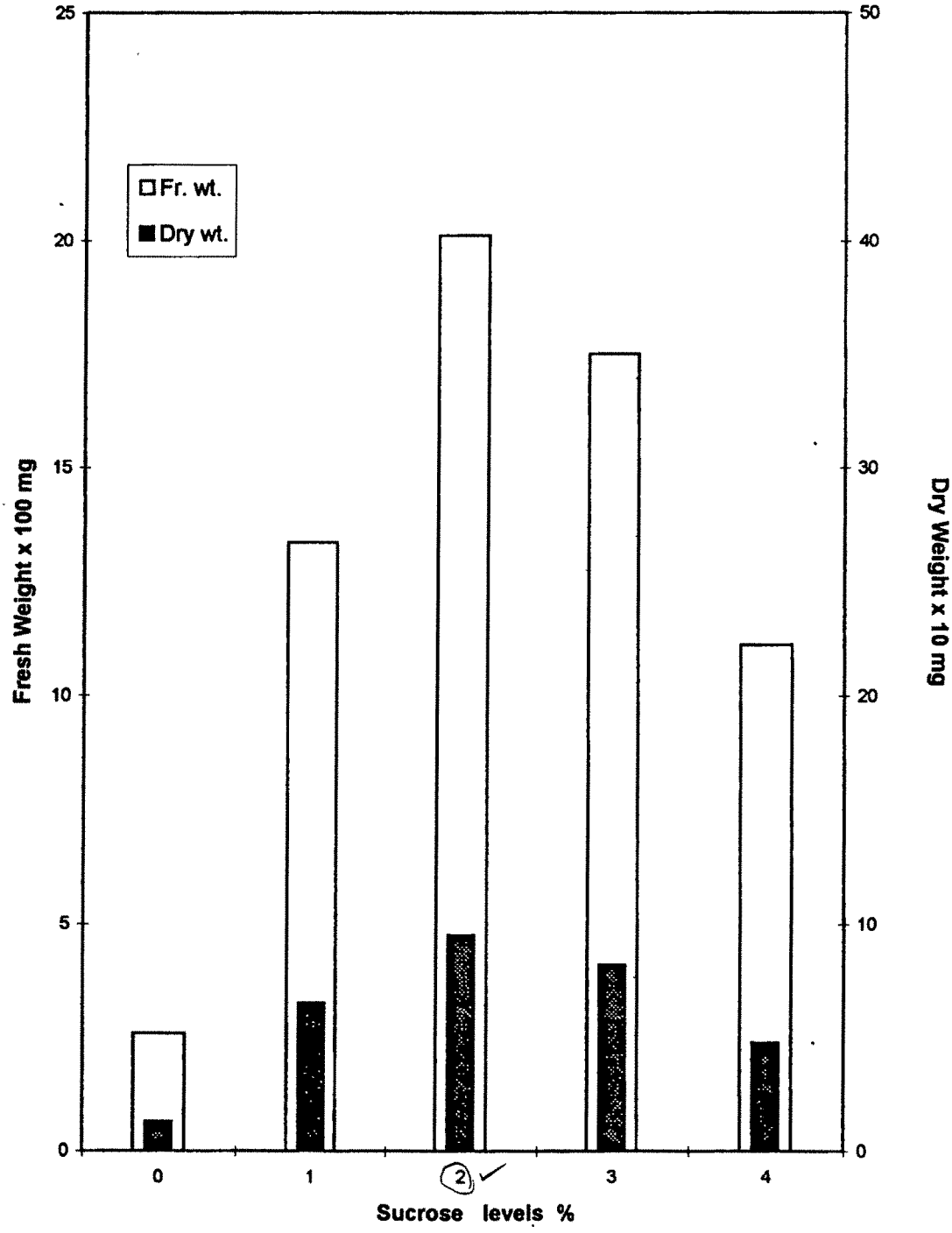

Fr. wt.
Dry wt.
Fresh Weight x 100 mg
Dry Weight x 10 mg
25
20
15
10
5
0
50
40
30
20
10
0
0
1
2
3
4
Sucrose levels %

Fig. 8

Experiment 11 : Growth kinetics of cultured roots

The growth pattern of excised roots grown in root culture medium (120 ml) was determined at weekly intervals upto four weeks and finally at the end of eight weeks. The culture roots were screened for their alkaloid (tropine) contents following the procedures described by Roberts and James (1947) as given in Chapter II 'Materials and Methods'.

The fresh and dry weights exhibited a linear increase in their amounts in the first two weeks as seen in Fig. 9. Thereafter, a sharp increase in the biomass values occurred at the end of three week culture period (the fresh and dry weights were 1800 ± 15 mg and 75 ± 3.6 mg respectively). The biomass production increased to its maximum at the end of four weeks (2100 ± 20 mg and 102 ± 3 mg fresh and dry weights). These values were 2200 ± 15 mg and 108 ± 5 mg at the end of eight weeks. The graph of the growth parameters produced a typical sigmoid curve.

Also the tropine contents of the cultured roots at regular weekly intervals when estimated, showed that in the first two weeks there was no synthesis / accumulation of the alkaloid in the roots. In the third week only its presence was detected. However, in the fourth week there was a slight accumulation of tropine ~~content~~ to the extent of 0.001% (Fig.9). By this time the colour of the roots had changed to brown (Fig. 10a). The roots after eight weeks turned dark brown (Fig. 10b) and their alkaloid contents improved (0.002%).

This experiment proved that the cultured roots retained the capacity for synthesis / accumulation of alkaloid (tropine).

Fig.9 Growth pattern of excised root in culture with the tropine contents

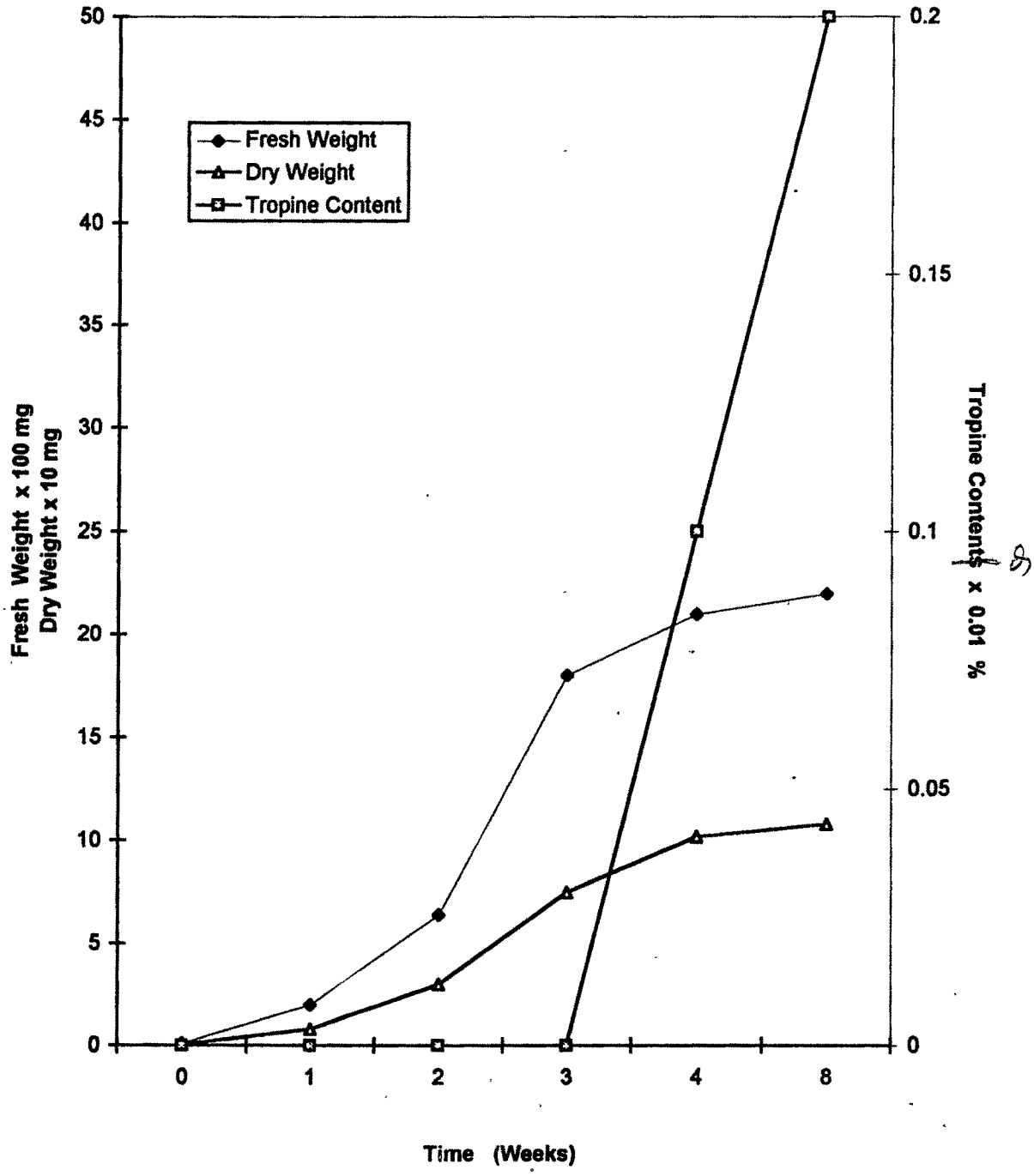

Fresh Weight
Dry Weight
Tropine Content
Fresh Weight x 100 mg
Dry Weight x 10 mg
Tropine Content x 0.01 %
Time (Weeks)
0
5
10
15
20
25
30
35
40
45
50
0
0.05
0.1
0.15
0.2
0
1
2
3
4
8

Fig. 9

Fig. 10 Excised roots in root culture medium

(a) After four weeks

(b) After eight weeks

a

b

Fig. 10

Experiment 12 : Effect of precursor (L - ornithine) feeding to the cultured roots

L-ornithine, being the precursor of tropane alkaloids, was supplemented to the root culture medium to find out its effect on the synthesis / accumulation of tropine alkaloid by the cultured roots. About 10 roots with tips (1-2 cm) were inoculated in root culture medium (40 ml) supplemented with L-ornithine at various levels (0-25 µ M/l). Culture flasks were maintained on gyratory shaker in culture room (25 ± 2° C) for four weeks.

Results recorded in Table XII show that with the incorportion of L-ornithine, the biomass values in terms of fresh and dry weights along with tropine contents increased linearly. At 15 µ M/l level of L-ornithine the fresh and dry weights were 2280 ± 17 mg and 119 ± 3 mg respectively with the tropine contents 0.04%. The white coloured excised roots turned yellowish in colour with distinct thickened portions (Fig. 11a). On further increase in L-ornithine level to 20 µ M/l level, the biomass reached to highest (2310 ± 12 mg and 125 ± 9 mg) in terms of fresh and dry weights. At the same time the cultured roots turned light brown (Fig. 11b). Besides, that these roots accumulated highest amount of tropine contents (0.15%). Further increase in L-ornithine level to 25 µ M/l level not only decreased the biomass production to 1800 ± 10 mg and 90 ± 3 mg in terms of fresh and dry weights respectively, but also dropped the tropine content to 0.02% (Table XII).

The cultured roots grown in 15 and 20 µ M/l level of L-ornithine were transferred to fresh medium after four weeks. At the end of eight weeks,

Table XII : Effect of L-ornithine on tropine synthesis / accumulation by cultured roots

Medium : MS root culture medium + sucrose (2%) + IAA + IBA (2 µM/l each) + L-ornithine (0-25 µM/l)

Inoculum : 10 roots with tips
Fresh weight - 10 ± 0.6 mg
Dry weight - 0.4 ± 0.02 mg

Incubation : 4 weeks in culture room at 25 ± 2° C in 16 h photoperiod (1000 lux)

Sr. No.	Levels of L-ornithine µM/l	Fresh wt. mg	Dry wt. mg	% tropine *
1	00	2150 ± 10	105 + 4	0.002
2	5	2240 ± 13	109 ± 4	0.008
3	10	2265 ± 15	112 ± 3	0.02
4	15	2280 ± 17	119 ± 3	0.04
5	20	2310 ± 12	125 ± 9	0.15
6	25	1800 ± 10	90 ± 3	0.02

Mean of six replicates with standard deviation

* % dry weight basis.

Fig. 11 Excised roots grown in

(a) 15 μ M/l L-ornithine

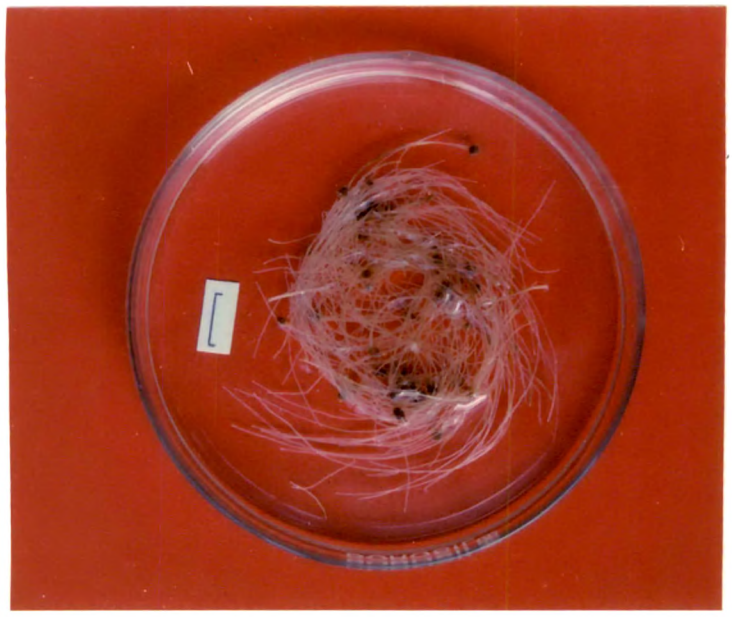
(b) 20 μ M/l L-ornithine

After four weeks

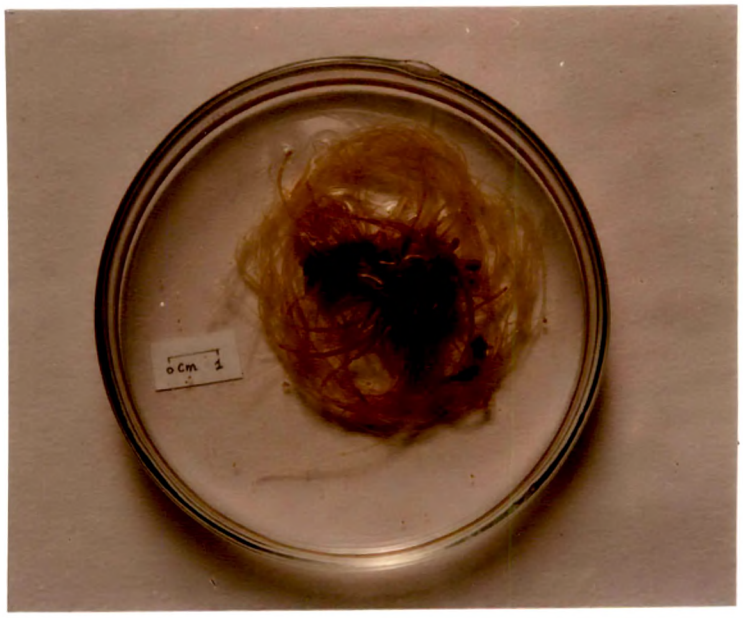
(c) 15 μ M/l L-ornithine

(d) 20 μ M/l L-ornithine

After eight weeks

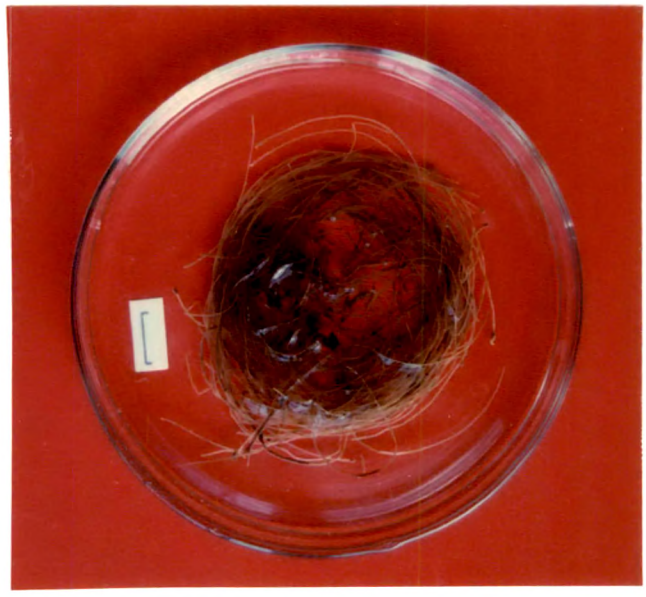


a

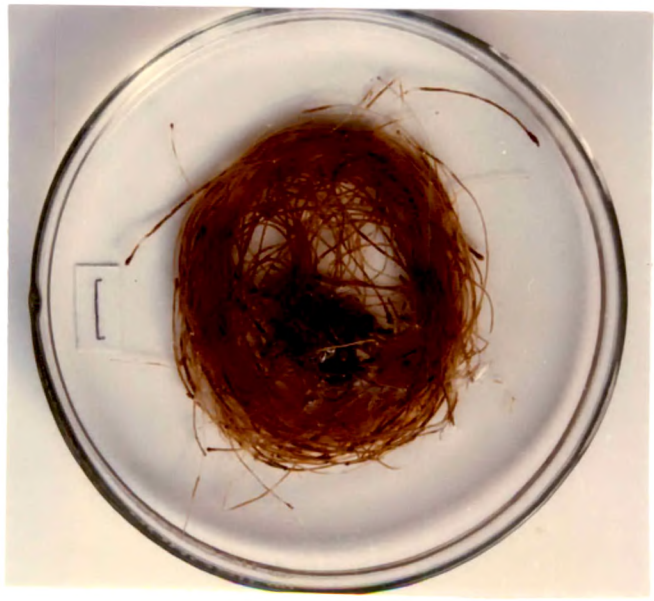


b

Fig. 11



c



d

Fig. 11

the biomass values recorded for 15 and 20 μM/l levels of L-ornithine were 2350 ± 20 mg and 2500 ± 15 mg, 115 ± 6 mg and 140 ± 8 mg in terms of fresh and dry weights respectively. Roots became dark brown in colour (Fig. 11c, d) with their tropine contents increasing to 0.07% and 0.35% respectively.

Thus, 20 μM/l of L-ornithine was the optimal level for tropine synthesis/accumulation in cultured roots.

(C) CALLUS CULTURES

The experimental work conducted for the successful establishment of callus cultures raised from various organs of the aseptically germinated *W. somnifera* plant has been described in this section. The medium used was Murashige and Skoog's (1962) along with a source of carbohydrate and requisite doses of phytohormones. In order to develop sufficient quantities of callus biomass, nutritional/hormonal requirements were standardised. Besides, the biosynthetic potential of these tissues for alkaloid synthesis/ accumulation was also examined.

Experiment 13 : Establishment of callus cultures from *W. somnifera* plant organs

Plants of *W. somnifera* were raised aseptically from seeds of 'elite' plant in petri dishes and individual organs *viz.*, hypocotyl, stem and leaf explants were excised and inoculated on the culture media. MS medium (20/30 ml) in test tubes/flasks containing sucrose (2%) supplemented with Kn (0-4 μM/l) alone, and in combination with IAA/NAA/2,4-D (0-8 μM/l), gelled with agar (0.8%) were used. Six replicates for each treatment were

maintained in the culture room at 25 ± 2°C in 16h photoperiod (1000 lux).

Results recorded in Table XIII during four week culture period showed that in the absence of Kn or in its presence at various levels, no callus initiation occurred from hypocotyl/stem/leaf explants except that the explants remained green for a short period in the presence of high doses of Kn. Addition of IAA to Kn-containing media at all levels failed to induce callus from hypocotyl/stem/leaf explants as evident from the results recorded in Table XIII.

The second auxin NAA was tested in combination with Kn at various levels to induce callus from these explants. In hypocotyl explants Kn at 1 μM/l with 2 μM/l of NAA induced white callus at the end of three weeks. Further increase either in Kn or NAA levels failed to induce the callus tissue.

In the presence of Kn and 2,4-D at 1 μM/l each, hypocotyl segments initiated yellowish callus within one week (Fig.12a) which turned brown with passage of time. Further increase either in Kn or 2,4-D levels failed to initiate callus earlier from hypocotyl explants as seen in the results of Table XIII.

Stem explants treated with Kn in combination with NAA at the levels tested failed to induce callus within four weeks period.

Leaf explants treated with Kn at 1 μM/l in combination with NAA at 2 μM/l induced slight callus from the midrib region. Further increase in Kn level to 2 μM/l failed to induce callus at faster rate. Increase in NAA

Table XIII : Callus induction from hypocotyl / stem / leaf explants of *W. somnifera* (L.) Dunal
Medium : M S + sucrose (2%) + Kn (0-4 µM/l) ± IAA / NAA / 2, 4-D (0 - 8 µM/l
Incubation :4 weeks in culture room at 25 ± 2°c in 16 h photoperiod (1000 lux)

Sr. No.	Phytohormones µM/l Kn	Hypocotyl					Stem					Leaf				
		0.0	0.5	1.0	2.0	4.0	0.0	0.5	1.0	2.0	4.0	0.0	0.5	1.0	2.0	4.0
		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1	IAA 0-8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2	NAA 0-8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3	0.5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4.	1.0	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-
5.	2.0	-	-	+	+	-	-	-	-	-	-	-	-	+	+	-
6.	4.0	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-
7.	6.0	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-
8.	8.0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9.	2,4-D 0.0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
10.	0.5	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-
11.	1.0	-	+	++++	+	+	-	-	-	-	-	-	+	+	+	-
12.	2.0	-	+	+	+	+	-	-	-	-	-	-	+	+	+	+
13	4.0	-	+	+	+	-	-	-	-	-	-	-	+	+	+++	+
14	6.0	-	-	-	-	-	-	-	-	-	-	-	+	+++	++++	+
15.	8.0	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+

Means of six replicates
- No response
+ sign denotes amount of callus induced

Fig. 12 Callus (C)induction from

(a) Hypocotyl segment

(b) Leaf segment

a

b

Fig. 12

levels to 4 µM/l did not improve callus induction; however, at 6 µM/l it was enhanced to appreciable ~~amount~~.

In the presence of Kn with 2,4-D, leaf segments induced white coloured callus when Kn levels were 0.5, 1.0 and 2.0 µM/l, with 2, 4-D levels at 1,2,4,6 and 8 µM/l. Amongst these, Kn at 2 µM/l and 2,4-D at 6 µM/l were the optimal levels observed, as callus ~~initiated~~ was induced in large quantities within a short period as seen in the results of Table ~~XIII~~ 17 and in Fig. 12b. Callus initiated from the midrib portion ~~and~~ proliferated all over the explants within a four weeks period.

Results of this experiment proved that Kn combined with 2,4-D at 1 µM/l ~~combination~~ was suitable for hypocotyl callus induction, ~~while~~ whereas Kn at 2 µM/l with 6 µM/l ~~level~~ of 2,4-D was ideal for leaf callus induction.

Stem callus could not be induced at the concentrations of Kn and 2,4-D ~~levels~~ tested. Hence, further experimental work was conducted on hypocotyl/leaf callus tissues ~~induced~~.

Experiment 14 : Biomass production of callus ~~tissues~~

(a) Effect of Kn levels

Healthy callus tissues (300 ± 20 mg Fr. wt.) of hypocotyl and leaf were transferred to MS medium (30 ml) containing sucrose (2%), Kn (0-4 µM/l) supplemented with 2,4-D at 1 µM/l for hypocotyl and 6 µM/l for leaf, respectively.

Results observed after four weeks showed that in the absence of Kn, hypocotyl and leaf calli registered poor biomass production in terms of

fresh and dry weights (Fig. 13). With the incorporation of Kn in the medium there was an enhancement in the biomass. The fresh and dry weight values reached their maximum at 3340 ± 50 mg and 168 ± 11 mg respectively when Kn level was 1 μM/l for hypocotyl, whereas these values for leaf were 4117 ± 55 mg and 200 ± 12 mg at 2 μM/l. Further increase in Kn levels lowered the biomass values for both the callus tissues (Fig.13).

Thus, it was evident that Kn at 1 μM/l for hypocotyl callus and Kn at 2 μM/l for leaf callus were the optimal levels for their respective biomass production.

(b) Effect of 2,4-D levels

In this experiment, MS media (30ml) containing sucrose (2%), Kn at 1 μM/l and 2 μ M/l individually, were supplemented with 2,4-D at various levels (0-8 μM/l). Culture vessels were inoculated with 300 ± 20 mg of fresh hypocotyl/leaf callus tissues.

Results depicted in Fig. 14 showed that in the absence of 2,4-D, no growth in terms of fresh and dry weights of both these callus cultures was observed. With the incorporation of 2,4-D in the medium the growth of callus tissues was resumed. There was a steady increase in the biomass production with the corresponding increase in the 2,4-D levels. The highest biomass values of 4420 ± 50 mg and 221 ± 18 mg; 4788 ± 47 mg and 240 ± 21 mg were achieved for hypocotyl and leaf calli respectively when 2,4-D was at 6 μM/l level. Beyond this level of 2,4-D the growth values declined.

Thus, it was proved that 2,4-D at 6 μM/l was the optimal level for maximum biomass production of both the callus tissues.

Fig. 13 Growth of hypocotyl and leaf callus tissues at various levels of Kn (0-4 μM/l)

Culture media for hypocotyl callus

MS + S (2%) + 2,4-D (6 μM/l + Kn (0-4 μM/l)

Culture media for leaf callus

MS + S (2%) + 2,4-D (6 μM/l + Kn (0-4 μM/l)

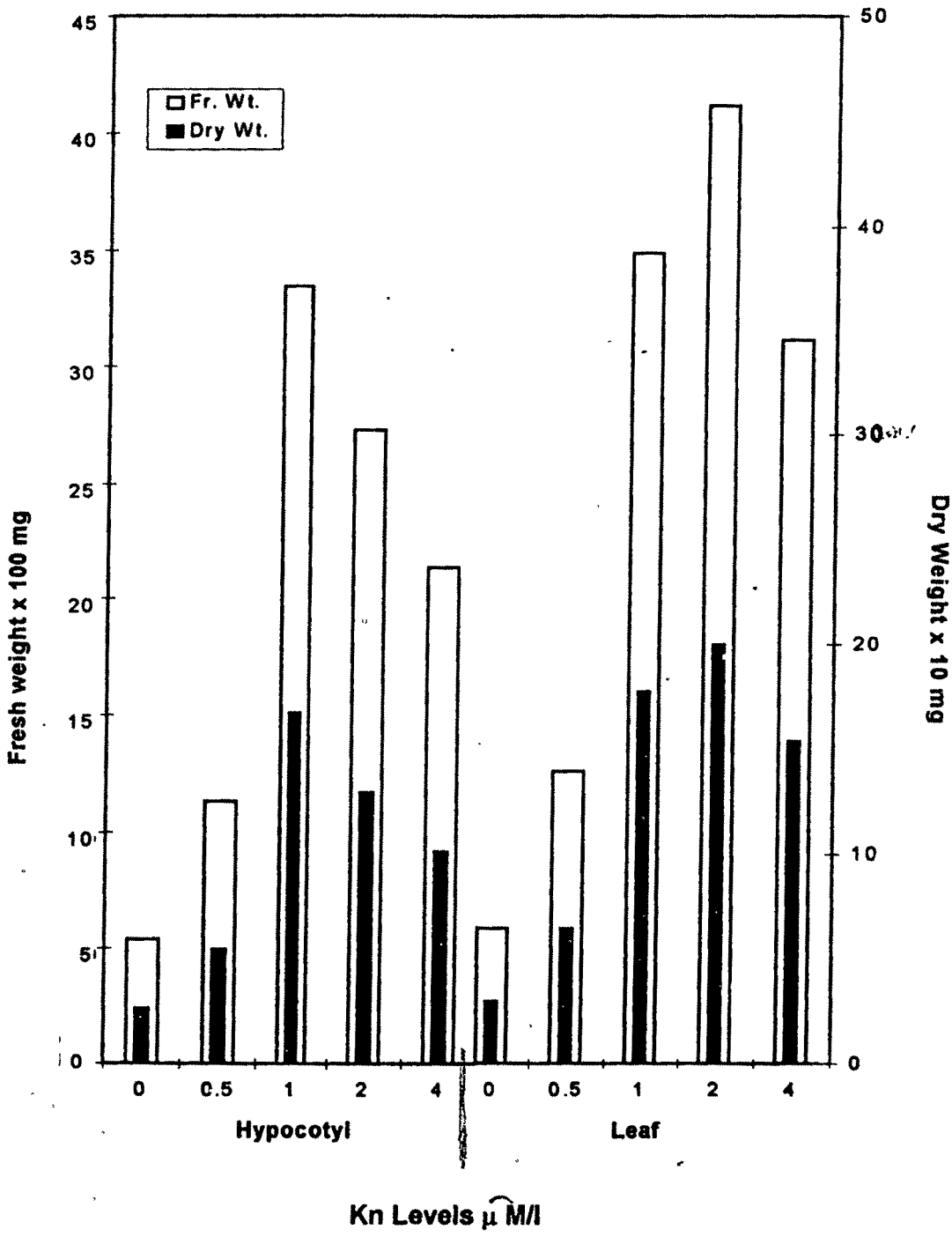

Fr. Wt.
Dry Wt.
Fresh weight x 100 mg
Dry Weight x 10 mg
45
40
35
30
25
20
15
10
5
0
50
40
30
20
10
0
0
0.5
1
2
4
0
0.5
1
2
4
Hypocotyl
Leaf
Kn Levels µ M/l

Fig. 13

Fig. 14 Growth of hypocotyl and leaf callus tissues at various levels of 2,4-D (0 to 8 μM/l)

Culture media for hypocotyl callus

MS + S (2%) + Kn (1 μM/l) +2,4-D (0 to 8 μM/l)

Culture media for leaf callus

MS + S (2%) + Kn (2 μM/l) + 2,4-D (0 to 8 μM/l)

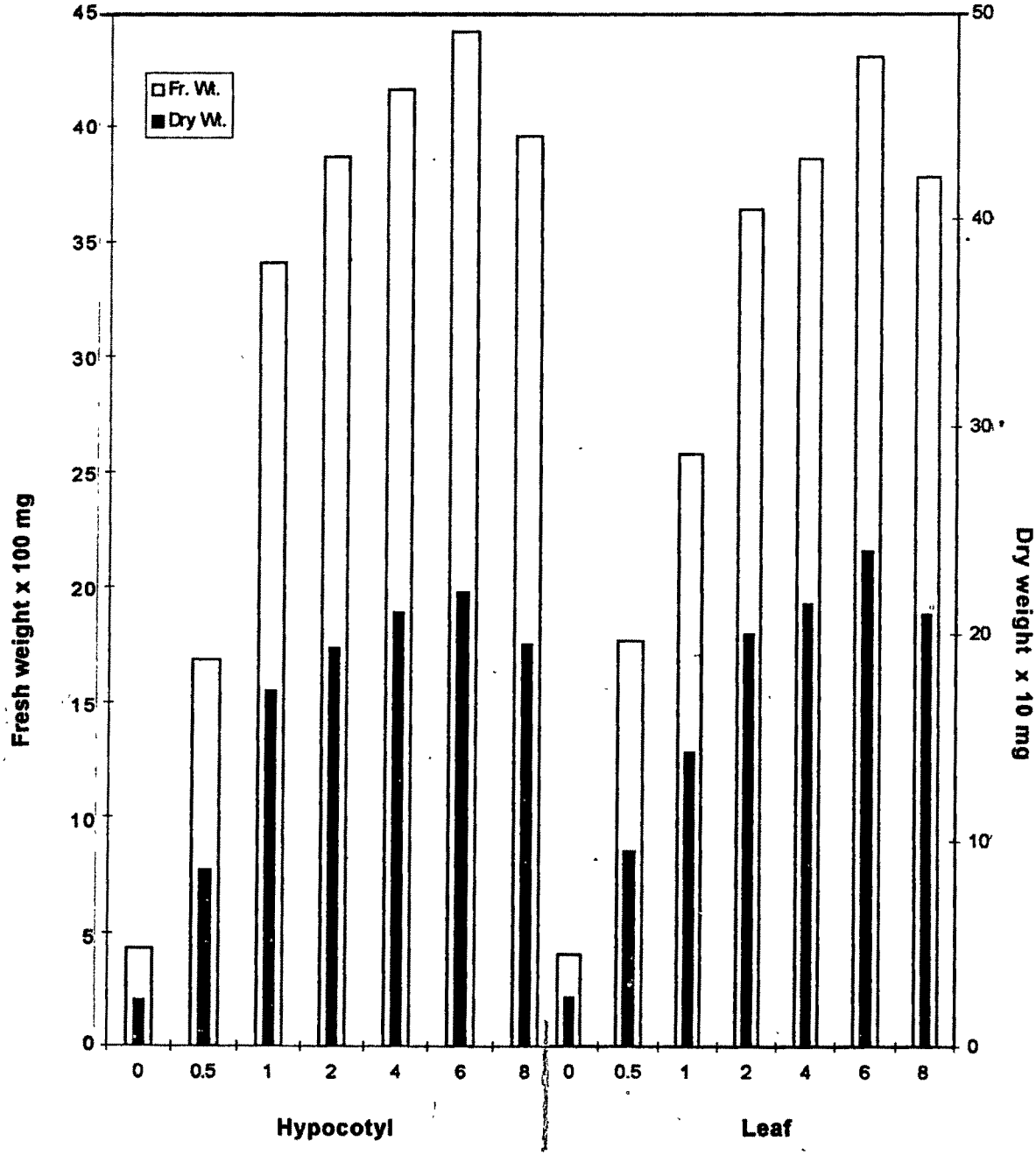

Fr. Wt.
Dry Wt.
Fresh weight x 100 mg
Dry weight x 10 mg
45
40
35
30
25
20
15
10
5
0
50
40
30
20
10
0
0
0.5
1
2
4
6
8
0
0.5
1
2
4
6
8
Hypocotyl
Leaf
2, 4-D Levels μ M/l

Fig. 14

Experiment 15 : Effect of various carbohydrates (Glucose / Sucrose / Mannitol) on callus tissues

MS medium (30 ml) containing 2,4-D (6 μM/l) with different concentrations of Kn (1 and 2 μM/l) supplemented with glucose/sucrose/ mannitol (2%) was inoculated with 300 ± 20 mg of fresh hypocotyl / leaf callus.

Results recorded in Table XIV indicated that hypocotyl and leaf calli in the presence of glucose ~~reached to~~ gave a biomass of 2470 ± 43 mg and 125 ± 9 mg; 2745 ± 38 mg and 138 ± 17 mg in terms of fresh and dry weights respectively. There was a variation in the type of callus tissues and (Fig.15a; Fig.16a) their growth was very slow.

Sucrose when substituted for glucose at the same concentration (2%) recorded faster growth of calli reaching their biomass values ~~to~~ of 4420 ± 50 mg and 221 ± 18 mg, 4788 ± 47 mg and 240 ± 21 mg ~~in terms of~~ fresh and dry weights respectively. Besides, ~~that~~ the callus tissues were friable in nature (Fig.15b; Fig.16b).

Incorporation of mannitol at 2% as the carbohydrate source in the medium recorded very poor growth of these tissues ~~as seen in the results of~~ (Table XIV) and they turned brown by the end of four weeks ~~period~~ (Fig.15c; Fig.16c).

~~Hence, results of~~ Thus this experiment proved that sucrose at 2% was superior to glucose and mannitol.

Table XIV: Effect of various carbohydrates (Glucose / Sucrose / Mannitol) on the growth of hypocotyl / leaf callus tissues

Medium : Hypocotyl callus
MS + Kn (1 µM/l)+ 2,4-D (6 µM/l)+ Glucose/ Sucrose / Mannitol (2%)

Leaf callus
M S + Kn (2 µM/l)+ 2, 4-D (6 µM/l) + Glucose/Sucrose / Mannitol (2%)

Inoculum : Fresh weight 300 ± 20 mg
Dry weight 15 ± 1 mg

Incubation : 4 weeks of culture period at 25 ± 2°C in 16h photoperiod (1000 lux)

Sr. No.	Carbohydrate 2%	Hypocotyl callus		Leaf callus	
		Fr. wt.	Dry wt.	Fr. wt.	Dry. wt.
1.	Glucose	2470 ± 43	125 ± 9	2745 ± 38	138 ± 17
2.	Sucrose	4420 ± 50	221 ± 18	4788 ± 47	240 ± 21
3.	Mannitol	635 ± 22	32 ± 8	825 ± 20	40 ± 13

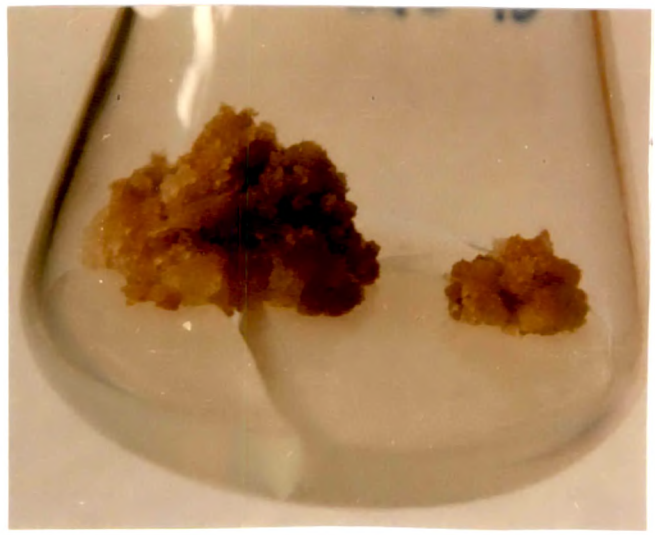
Mean of six replicates with standard deviation

Fig. 15 Hypocotyl callus grown on MS medium containing

(a) Glucose (2%)

(b) Sucrose (2%) - highest biomass production

(c) Mannitol (2%)



a

b

c

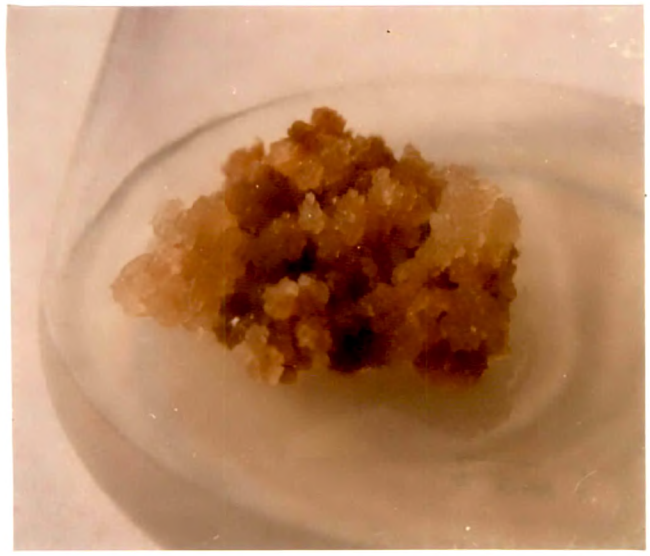
Fig. 15

Fig. 16 Leaf callus grown on MS medium containing

(a) Glucose (2%)

(b) Sucrose (2%) - highest biomass production

(c) Mannitol (2%)



a

b

c

Fig. 16

Experiment 16 : Determination of optimal sucrose level for biomass production

Hypocotyl/leaf callus tissues (300 ± 20 mg Fr. wt.) maintained on basal medium for a week and then transferred to MS medium (30 ml) containing Kn (1 µM/l) and Kn at (2 µM/l) in combination with 2,4-D (6 µM/l) M/l supplemented with sucrose at various levels (0-4%). Culture flasks were incubated as per procedures given in Chapter - II.

Results recorded (Fig.17) after four weeks period indicated that in the absence of sucrose, the callus tissues ceased to grow and it turned brown and ultimately died. Addition of sucrose at 1% in the medium induced the growth of callus tissues and their biomass values reached upto 3250 ± 40 mg and 163 ± 4 mg for hypocotyl callus, 3860 ± 30 mg and 192 ± 8 mg for leaf callus in terms of fresh and dry weights respectively. Maximum biomass production was achieved when sucrose level was 2%, and their fresh and dry weights were 4500 ± 47 mg and 225 ± 8 mg; 4770 ± 45 mg and 240 ± 10 mg for hypocotyl and leaf calli respectively. However, sucrose at concentration of 3% or above failed to improve the biomass values of these calli.

Thus, sucrose at 2% was found to be the optimal level for biomass production of hypocotyl and leaf callus tissues. Therefore, MS medium with 2% sucrose supplemented with Kn (1 µM/l) and 2,4-D (6 µM/l) was designated as standard medium for hypocotyl callus. MS medium with 2% sucrose supplemented with Kn (2 µM/l) and 2,4-D (6 µM/l) was designated as the standard medium for leaf callus.

Fig. 17 Growth of hypocotyl and leaf callus tissues at various levels of sucrose (0-4%)

Culture media for hypocotyl callus

MS + Kn (1 μM/l) + 2,4-D (6 μM/l) + S (0-4%)

Culture media for leaf callus

MS + Kn (2 μM/l) + 2,4-D (6 μM/l) + S (0-4%)

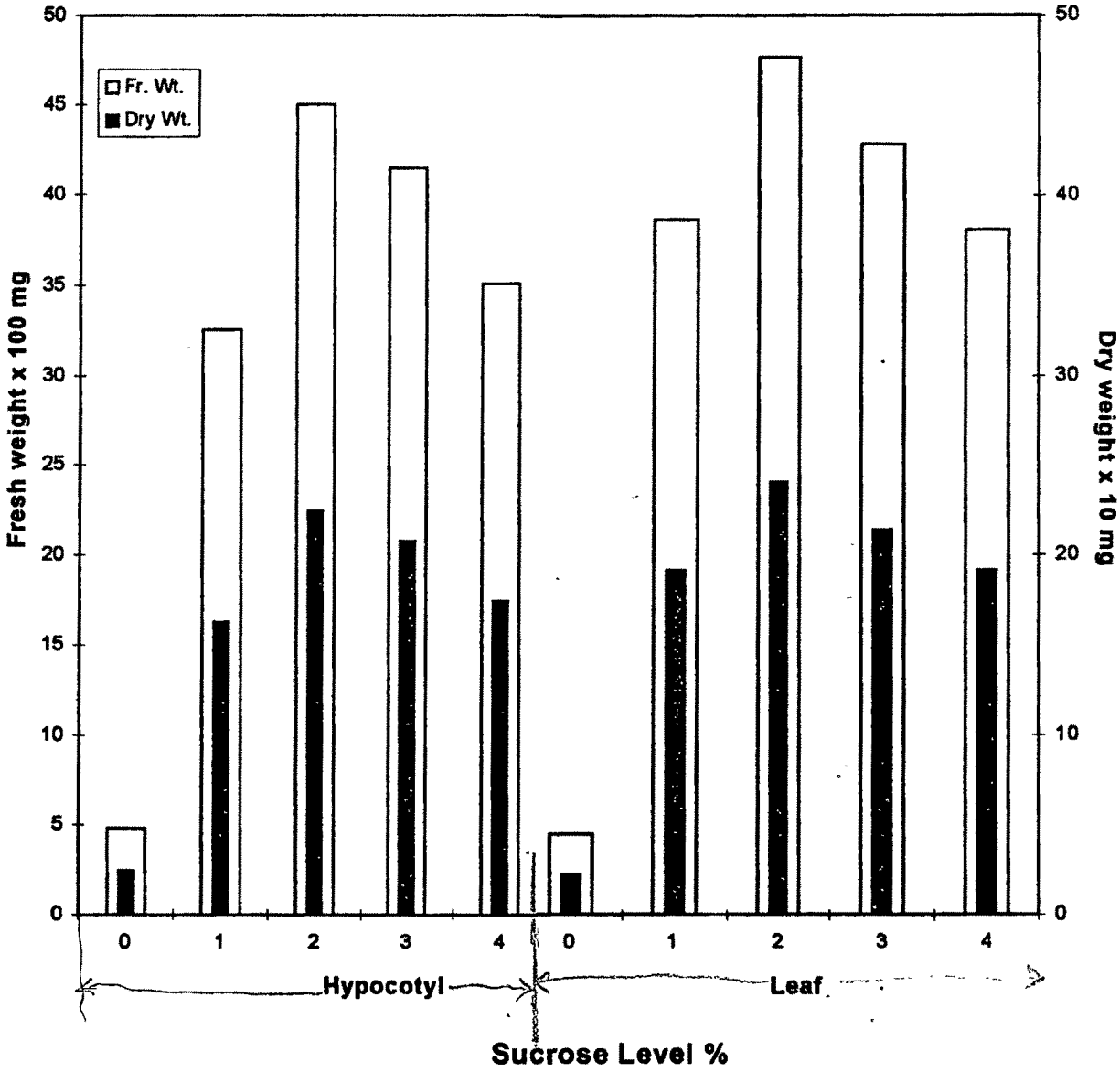

Fr. Wt.
Dry Wt.
Fresh weight x 100 mg
Dry weight x 10 mg
50
45
40
35
30
25
20
15
10
5
0
0 1 2 3 4 0 1 2 3 4
Hypocotyl
Leaf
Sucrose Level %

Fig. 17

Experiment 17 : Growth kinetics and Tropine profile of callus cultures

Healthy callus tissues (300 ± 20 mg Fr. wt.) were transferred to the respective standard media (30 ml) to find out their growth kinetics. Culture flasks were maintained in culture room at 25 ± 2° C for 16 h photoperiod (1000 lux).

(a) Growth kinetics

Results recorded at weekly intervals indicated that during the first week both the callus tissues showed slight growth which improved during the second and third week. The maximum growth values recorded were 4420 ± 50 mg and 4788 ± 47 mg; 200 ± 18 mg and 240 ± 21 mg for hypocotyl and leaf callus in terms of fresh and dry weights at the end of four weeks. These values at the end of the eight weeks incubation period were 4530 ± 12 mg and 5000 ± 35 mg; 211 ± 10 mg and 250 ± 12 mg respectively (Fig.18).

The graph of the growth pattern of both the callus tissues exhibited sigmoid curves indicating that they followed the normal growth.

(b) Tropine profile of hypocotyl/leaf callus tissues

Callus tissues of hypocotyl/leaf grown on standard medium were screened for their tropine contents at the end of four weeks. In hypocotyl callus the presence of tropine alkaloid was detected, while during the same time leaf callus accumulated 0.005% of the alkaloid (Fig.18). At the end of eight weeks period tropine contents of hypocotyl callus was 0.001% and of the leaf callus it was 0.018% (Fig.18). This means that the leaf callus showed enhacement of tropine contents during four to eight week period.

Fig. 18 Growth kinetics and tropine contents of hypocotyl and leaf callus tissues during eight weeks period

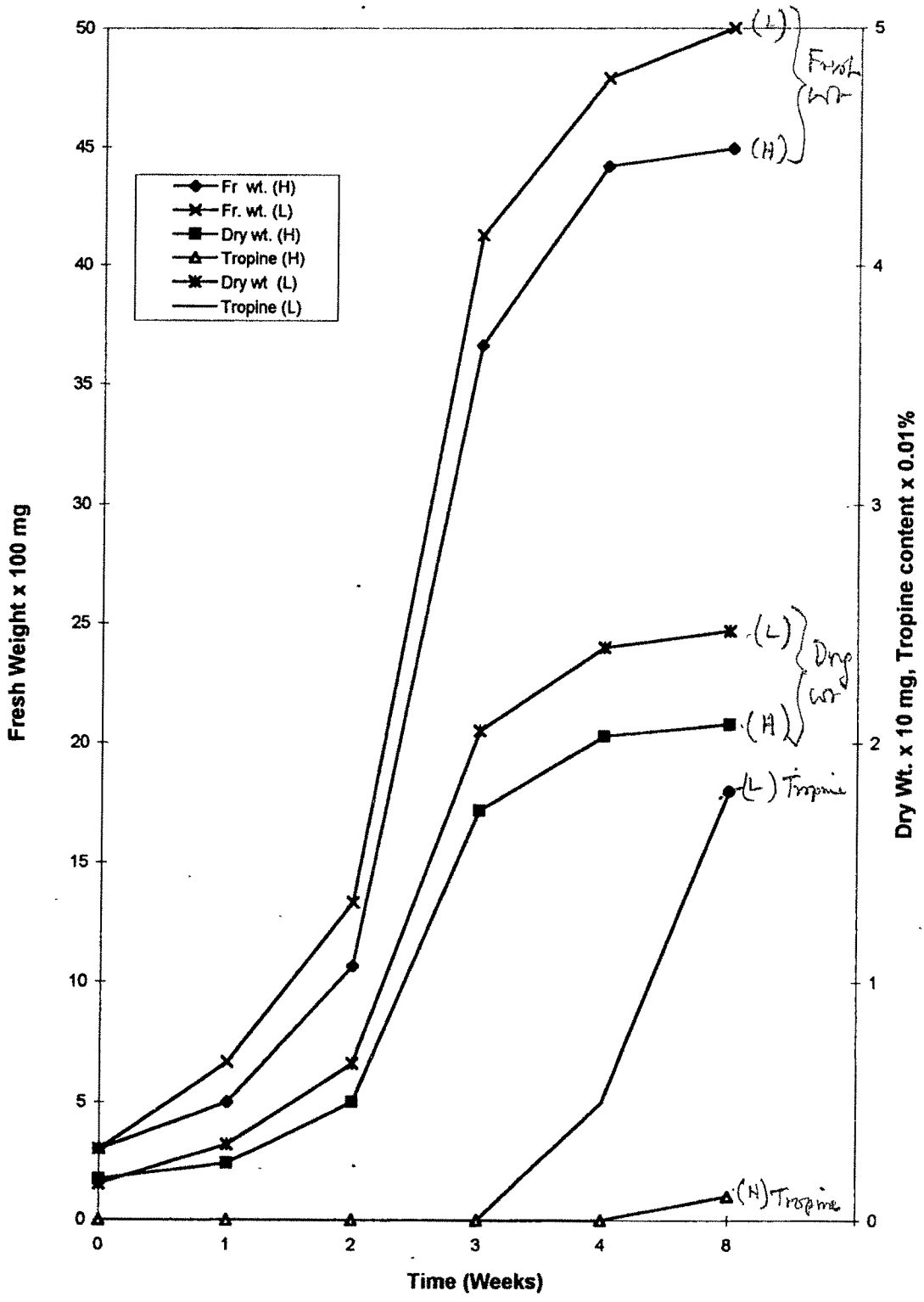

Fr wt. (H)
Fr. wt. (L)
Dry wt. (H)
Tropine (H)
Dry wt (L)
Tropine (L)
Fresh Weight x 100 mg
Dry Wt. x 10 mg, Tropine content x 0.01%
Time (Weeks)
0
1
2
3
4
8
0
5
10
15
20
25
30
35
40
45
50
0
1
2
3
4
5
(L)
(H)
(L)
(H)
(L) Tropine
(H) Tropine

Fig. 18

Thus, the experiment clearly proved that the leaf callus possessed excellent potential for tropine synthesis / accumulation as compared with the hypocotyl callus. Hence, further experimental work was carried on leaf callus tissues.

Experiment 18 : Studies on suspension cultures

(a) Establishment of suspension culture of leaf callus

In order to establish suspension cultures, healthy friable leaf callus pieces were transferred to Erlenmeyer flasks containing standard medium (25 ml) and the culture flasks were maintained on gyratory shaker (120 rpm) at 25 ± 2°C for 16 h photoperiod (1000 lux).

Within a week the callus separated into single cells and cell units due to the agitation caused. Each suspension culture was aseptically filtered through nylon mesh (45 µ pore) and 5 ml of the filtrate was subcultured in fresh medium of the same composition. Microscopic observations of the filtrate showed that it consisted mostly of single cells and cell units of 5-7 cells. Cells when stained with Evan's blue, 75% remained unstained indicating that they were the viable cells.

(b) Growth kinetics of suspension cultures

Cells in suspension cultures were harvested at weekly intervals and growth measurements in terms of fresh/dry weights, cell number, packed cell volume (PCV) and alkaloid content were determined as per the procedures described in Chapter II 'Materials and Methods'.

Results depicted in Fig.19 indicated that cell-biomass in the first week increased in a linear manner, recording their fresh and dry weights as 660 ± 8 mg and 30 ± 2 mg respectively, the cell number reached to 1.44 x 10^5 cells/ml and the PCV to 10%. Further, the biomass values showed a sharp rise in their fresh (4695 ± 32 mg) and dry (223 ± 10 mg) weights, the cell number increased to 3.96 x 10^5 cells/ml and packed cell volume to 30% (Fig.19) at the end of the third week. In the fourth week their values were slightly enhanced.

During this period, the tropine contents of suspension cultures were 0.002% at the end of the first week. After three weeks, there was a sharp increase (0.02%). But with the passage of time, tropine contents declined to 0.017% after four weeks period.

Experiment 19 : Effect of precursor - L-ornithine feeding on tropine contents of the suspension cultures

Cell suspension (5 ml) from stock cultures were transferred to liquid media (25 ml) with L-ornithine at various levels (5, 10, 15, 20, 25 μM/l) and control. Culture flasks were incubated for three weeks on gyratory shaker at 25 ± 2°C for 16 h photoperiod (1000 lux).

Results in Table XV indicated that the tropine contents of the suspension culture (control) was 0.02%. With the incorporation of L-ornithine at 5 μM/l in the culture medium, a sharp increase in the tropine contents to 0.022% was noticed. Further increase in the L-ornithine to 10 μM/l enhanced the tropine content to 0.025%, whereas it was 0.027% at 15 μM/l level. Highest

Fig. 19 Growth kinetics and tropine contents in cell suspension of leaf callus during four weeks.

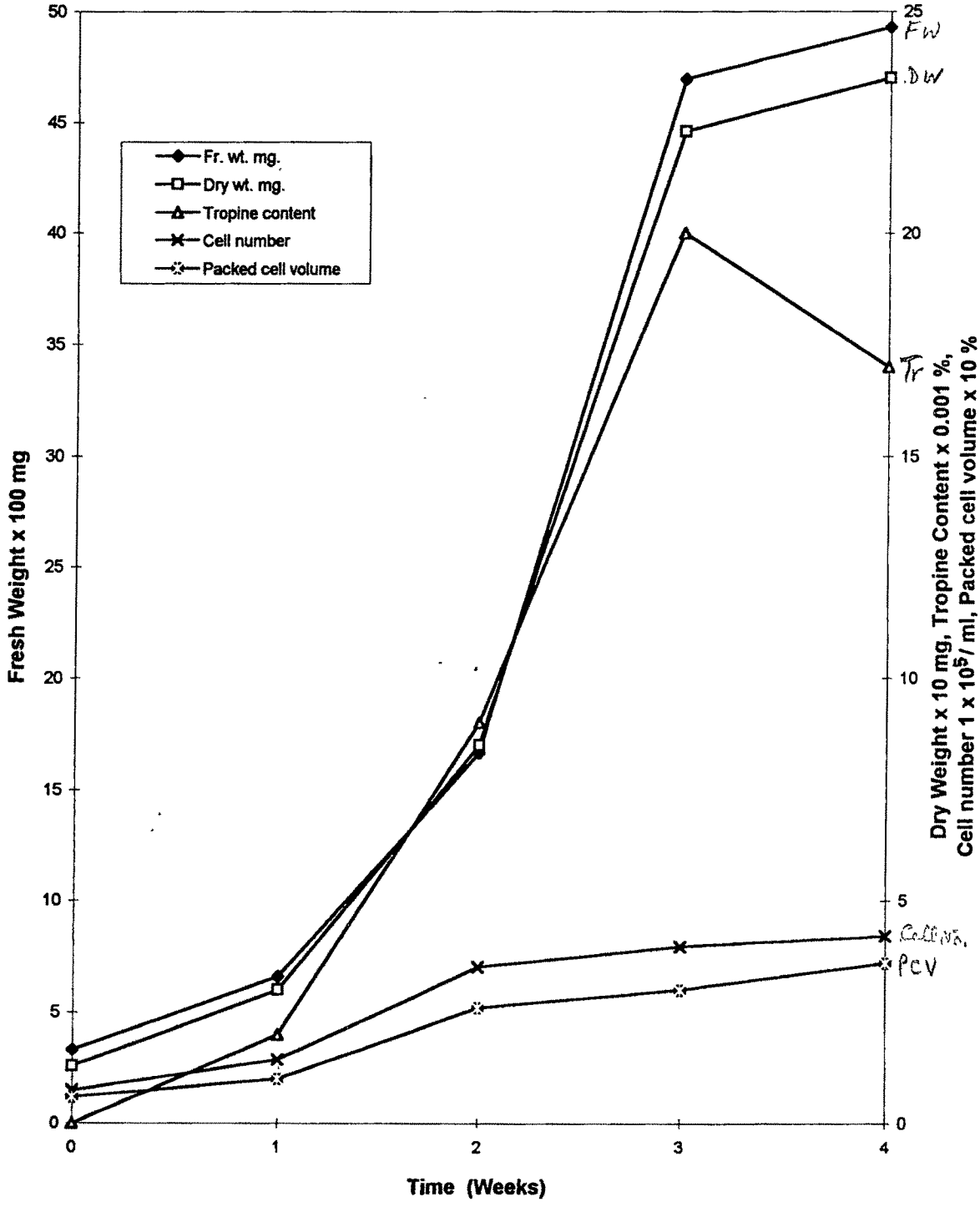

Fr. wt. mg.
Dry wt. mg.
Tropine content
Cell number
Packed cell volume
Fresh Weight x 100 mg
Dry Weight x 10 mg, Tropine Content x 0.001 %,
Cell number 1 x 10⁵/ ml, Packed cell volume x 10 %
Time (Weeks)
FW
DW
Tr
Cell no.
PCV
0
5
10
15
20
25
30
35
40
45
50
0
1
2
3
4

Fig. 19

Table XV: Effect of L-ornithine on growth and tropine synthesis of cells in suspension culture

Medium : MS + sucrose (2%) + Kn (2 μM/l) + 2,4-D (6 μM/l) + L-ornithine (5, 10, 15, 20, 25 μM/l))

Incubation : 3 weeks in culture room at 25 + 2°C in 16 h photoperiod (1000 lux)

Sr. No.	L-ornithine μM/l	Fr. wt. mg	Dry wt. mg	% * tropine
1.	Control	4695 ± 85	223 ± 11	0.020
2.	5	4200 ± 75	200 ± 10	0.022
3.	10	3910 ± 80	180 ± 15	0.025
4.	15	3845 ± 90	178 ± 8	0.027
5.	20	3657 ± 60	173 ± 10	0.030
6.	25	3600 ± 65	170 ± 12	0.015

Mean of six replicates with standard deviation

*% dry weight basis

tropine content of 0.030% was recorded at 20 µ M/l level. Further rise in L-ornithine level to 25 µ M/l declined the synthesis / accumulation of tropine of cell suspension culture to 0.015%. It was interesting to note that with the addition of precursor to the culture medium, the biomass production was somewhat reduced. At the same time, the tropine contents were enhanced with the incorporation of L-ornithine.

Thus, it was concluded that exogenous feeding of L-ornithine to the cells in suspension improved the synthesis / accumulation of tropine contents.

Experiment 20 : Selection of high yielding cell-lines

In this experiment cell-suspension (2 ml) was plated on standard medium (15 ml). Petriplates were incubated in culture room at 25 ± 2°C for 16 h photoperiod.

Results recorded showed that cell colonies were developed (Fig.20) and their tropine contents were 0.025%. Cells of the colonies were aseptically transferred to liquid medium and fresh suspension was prepared. This suspension was cultured once again, plated, and the tropine content increased to 0.038%. Hence, the effect of precursor feeding was most effective during two to three cultures.

This experiment showed that high-yielding cell lines could be established by feeding L-ornithine to the suspension cultures.

Fig. 20 Colony formation from cell-suspension on standard medium

After four weeks

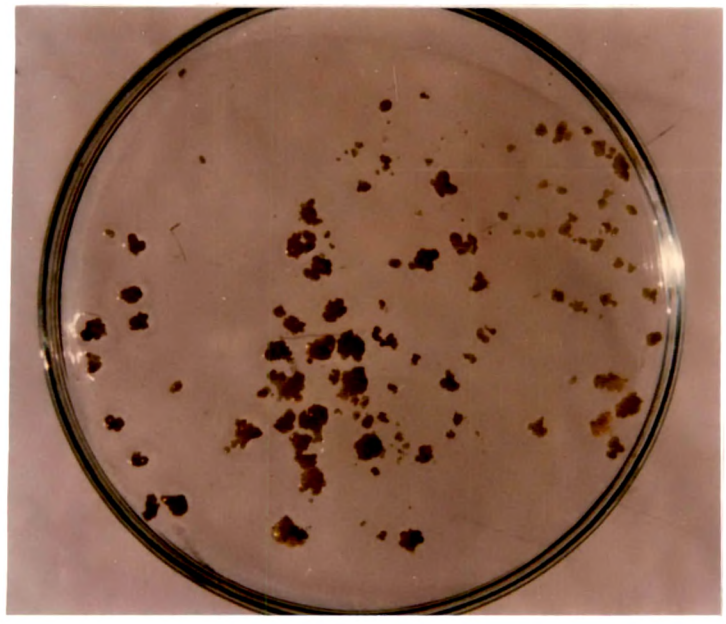


Fig. 20

Experiment 21 : Morphogenetic potential of callus cultures

The callus tissues grown on standard medium were transferred to basal medium for a week before they were subjected to sucrose 3% with Kn at 2 μM/l in combination with IAA/NAA/2,4-D (0.1 - 0.6 μM/l). Callus pieces of 1-2 cm in diameter were transferred to the above mentioned media and the flasks were kept in culture room at 25 ± 2°C in 16 h light (1000 lux).

Results showed (Table XVI) that callus treated with IAA (0.1 to 0.4 μM/l) differentiated only roots (60-80%) within two weeks (Fig.21a). The callus failed to show any response when IAA level was increased to 0.6 μM/l.

No morphogenic response was evoked in the presence of NAA from the callus cultures.

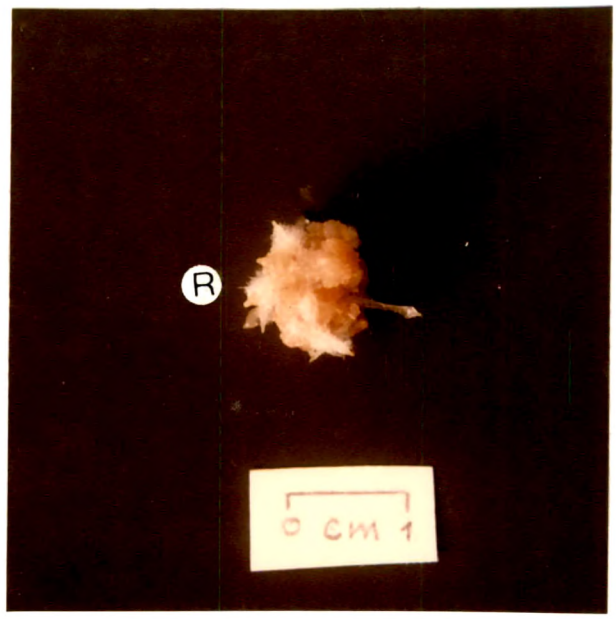
White In the presence of 2,4-D (0.1 - 0.4 μM/l) in the culutre medium, the callus turned nodular (50-95%) within four weeks (Fig. 21b).

Histological observations of each nodule revealed that it consisted of meristematic growth centres formed of thin walled cells which were concentrically arranged, with prominent nuclei (Fig. 22 a,b)

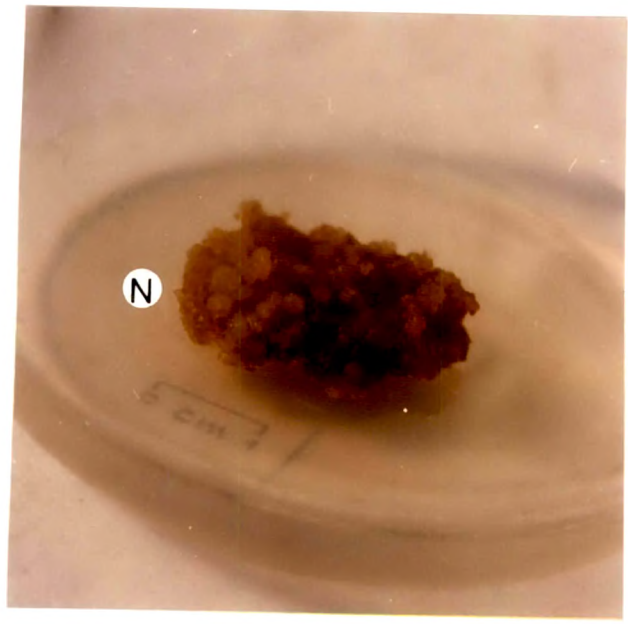
Results recorded in Table XVII indicate that when Kn at 1.0 μM/l in combination with BAP from 1 to 6 μM/l were added to the culture media, failed to evoke any morphogenic response. With increase in Kn level to 2 μM/l and BAP at 2 μM/l, the nodular callus turned green (100%). At the same level of Kn, with increase in BAP to 4 and 6 μM/l, nodular callus

Fig. 21 (a) Roots (R) differentiated from callus grown on media containing IAA (0.1 - 0.4 μM/l)

(b) Nodular callus (N) in after two weeks



a



b

Fig. 21

Table XVI: Differentiation of callus tissues of *W. somnifera* (L.) Dunal

Medium : M S + sucrose (3%) + Kn (2μ M/l) + IAA / NAA / 2,4-D(0.1-0.6μ M/l)

Incubation : 4 weeks in culture room at 25 ± 2°C in 16 h photoperiod (1000 lux)

Sr. No.	Auxin (μ M/l)	Morphogenic response	% response
	IAA		
1	0.1	R	80
2	0.2	R	60
3	0.4	R	80
4	0.6	-	-
	NAA		
5	0.1	-	-
6	0.2	-	-
7	0.4	-	-
8	0.6	-	-
	2, 4-D		
9	0.1	NC	95
10	0.2	NC	75
11	0.4	NC	50
12	0.6	-	-

Mean of six replicates

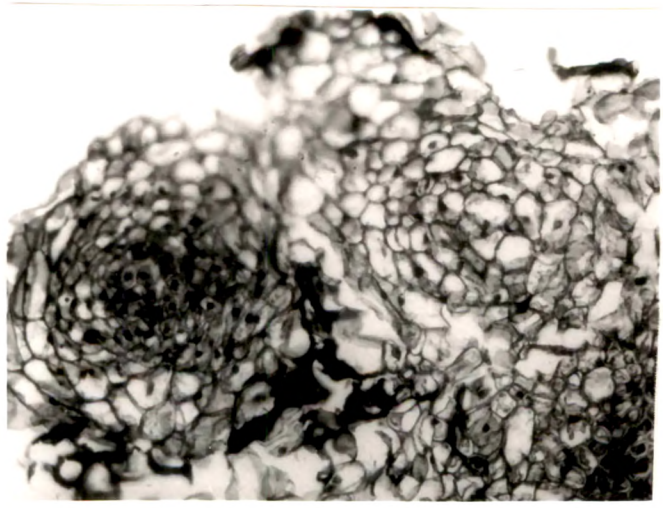
- No response

R - Roots

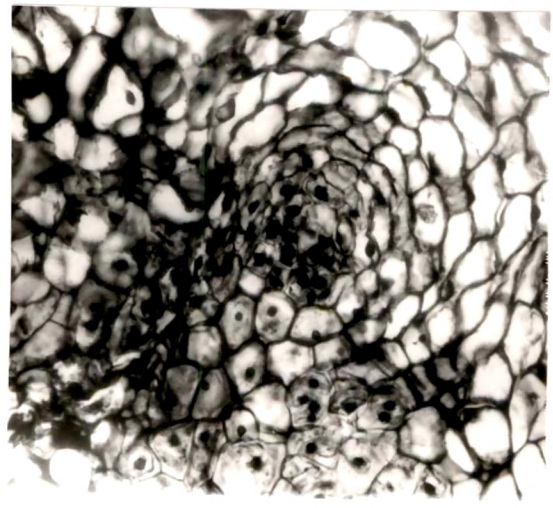
NC- Nodular callus

Fig. 22 (a) T. S. of nodule showing meristematic growth centres (160 X)

(b) Single nodule showing cells with prominent nuclei (250 X)



a



b

Fig. 22

differentiated into shoot buds (80-90%). Increase in Kn level to 4 µM/l with BAP at 1 and 2 µM/l, the callus remained green. Keeping Kn constant and increasing BAP to 4 µM/l induced 15-17 shoot buds (Fig. 23) with 95% response, whereas at 6 µM/l of BAP, the number of shoot buds decreased to 4-5 (80%). Increase in Kn level to 6 µM/l with BAP at 1 and 2 µM/l, turned the callus green, and it differentiated into 7-8 shoot buds (85%). At 4 µM/l and 6 µM/l of BAP no morphogenic response was observed.

This experiment proved that Kn and BAP each at 4 µM/l level induced highest number of shoot buds as seen in Table XVII.

The shoot buds were transferred to fresh medium of the same composition and allowed to grow for four weeks.

Rooting of these shoot buds was achieved by transferring them to MS medium with sucrose (1%) supplemented with IBA at 1 µM/l level where it grew well and developed roots (Fig.24).

Experiment 22 : Regenerative potential of excised leaves

This experiment was conducted to find out the regenerative potential of excised leaves. First to fourth leaves from the apex of a branch in serial order of development were used as the explants. They were cultured on MS basal medium as well as other media supplemented with IAA/IBA (0.5 - 4 µM/l). Experimental procedures were maintained as given in Chapter II 'Materials and Methods'.

First to third leaves and their segments cultured on all the test media failed to regenerate any organs and turned white within four weeks.

Table ~~XVII~~ : Shoot bud differentiation from leaf callus

Medium : M S + sucrose (3 %) + Kn (1-6 µ M/l) + BAP (1-6 µ M/l)

Incubation : 4 weeks in culture room at 25 ± 2°C in 16 h photoperiod (1000 lux)

Sr. No.	Cytokinin level µ M/l	Morphogenic response of nodular callus	Number of shoot buds	% response
	Kn + BAP			
1	1 1	-	-	-
2	1 2	-	-	-
3	1 4	-	-	-
4	1 6	-	-	-
5	2 1	-	-	-
6	2 2	Green	-	100
7	2 4	Shoot buds	7 - 8	90
8	2 6	Shoot buds	3 - 4	80
9	4 1	Green	-	80
10	4 2	Green	-	80
11	4 4	Shoot buds	15 - 17	95
12	4 6	Shoot buds	4 - 5	80
13	6 1	Green	-	90
14	6 2	Green	-	60
15	6 4	Shoot buds	7 - 8	85
16	6 6	-	-	-

Mean of six replicates

-No response

Fig. 23 Regenerated shoots (15-20) from callus

Fig. 24 Plantlet developed from a single shoot

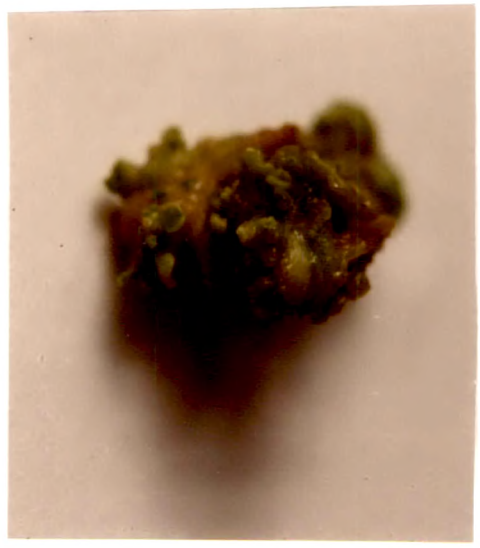


Fig. 23

Fig. 24

Results recorded in Table XVIII indicate that the fourth leaf when cultured on basal medium also failed to regenerate either roots or shoots. However, when IAA at 0.5 μM/l was incorporated in the medium, a few roots developed. With increase in IAA level to 1 and 2 μM/l 2-3 roots (80%) could be regenerated whereas at 4 μM/l the regenerative potential of leaf to regenerate roots was lost.

Excised leaves cultured on IBA at 0.5 μM/l regenerated 6-8 roots (80%) and the number of roots increased to 15-20 (90%) at 1 μM/l level (Fig.25). With further increase in IBA level to 2 and 4 μM/l the number of roots was reduced.

The second type of morphogenetic response was observed in the fourth leaf when it was cultured on MS media supplemented with Kn and BAP (1 to 6 μM/l).

Results recorded in Table XIX indicate that with the addition of Kn at 1 μM/l in combination with BAP (1-6 μM/l) failed to induce morphogenic response. However, Kn at 2 μM/l level in combination with BAP at 4 μM/l induced 5-6 shoot buds (Table XIX). Keeping Kn constant and increasing BAP to 6 μM/l did not evoke any response. Increase in Kn to 4 μM/l in combination with BAP at 2 μM/l induced 6-7 shoot buds and the number of shoot buds were enhanced to 10-12 (Fig. 26) at 4 μM/l level. Further rise in BAP level to 6 μM/l with the same concentration of Kn decreased the number of shoot buds.

This experiment has proved that Kn and BAP each at 4 μM/l level induced highest number of shoot buds as seen in Table XIX.

Table XVIII : Regeneration of roots from the fourth leaf segments of *W. somnifera*.

Medium : M S + sucrose (2%) + IAA / IBA (0.5 - 4 µM/l)

Inoculum : Leaf segments of the fourth leaf

Incubation : 4 weeks in culture room at 25 ± 2°C in 16 h photoperiod (1000 lux)

Sr. No.	Levels of Auxin (µM/l)	Number of regenerated roots	% response
1	0.0	-	-
	IAA		
2	0.5	1 - 2	80
3	1.0	2 - 3	80
4	2.0	1 - 2	80
5	4.0	-	-
	IBA		
6	0.5	6 - 8	80
7	1.0	15 - 20	90
8	2.0	9 -12	80
9	4.0	4 - 6	60

Mean of six replicates

- No response

Fig. 25 Roots regenerated (R) from midrib of cultured leaf segment

Fig. 25

Table XIX : Shoot-bud/s induction from cultured excised leaf

Medium : MS + sucrose (3%) + Kn (1 - 6 µ M/l) + BAP (1 - 6 µ M/l)

Incubation : 4 weeks in culture room at 25 + 2° C in 16 h photoperiod (1000 lux)

Sr. No.	Cytokinin level (µ M/l)		Morphogenic response No. of Shoot buds/explant
	Kn	**+ BAP**	
1	1	1	-
2	1	2	-
3	1	4	-
4	1	6	-
5	2	1	-
6	2	2	-
7	2	4	5-6
8	2	6	-
9	4	1	-
10	4	2	6-7
11	4	4	10-12
12	4	6	3-4
13	6	1	-
14	6	2	-
15	6	4	-
16	6	6	-

Mean of six replicates

- No response

Fig. 26 Shoot buds (10-12) from midrib of the leaf (lower surface)

Fig. 27 Shoot buds developed into plantlets directly?

Fig. 26

Fig. 27

In order to find out the ability for plantlet formation, they were detached from the explant and cultured on fresh medium of the same composition. Within four weeks these shoot buds produced plantlets as seen in Fig.27.

Rooting of these shoot buds was achieved by transferring them to sterilized vermiculite containing pots. Six to eight plants were well developed and were transferred to field conditions where they are growing well.

(D) PRODUCTION OF ANDROGENIC HAPLOIDS

This section describes the experimental work for the production of haploids in *W. somnifera* (L.) Dunal. The necessary conditions required for the raising of haploids *viz.*, age of buds, developmental stage of the pollens and nutritional requirements were standardised.

Experiment 23 : Selection of suitable age of bud for induction of haploids

a) Age of bud

The seeds of single fruits of elite *W.somnifera* plant (Fig. 28a) were germinated and plantlets raised in pots (Fig. 28b). As soon as the flowering set in, the floral buds were collected from axillary cyme inflorescence (Fig. 28c). The buds were dissected out from the youngest to the oldest and the developmental stage of pollens was recorded according to procedures described in chapter II Materials and Methods.

Results recorded in Table 24 indicate that the youngest floral bud contained pollen mother cells (PMC) in dividing condition. The next bud

Fig. 28 (a) Ripe fruits of *W. somnifera* growing in Ellora Park area

(b) Plants raised from above fruits

(c) Inflorescence

a

b

c

Fig. 28

showed pollen tetrads and 5% of the pollens were in the uninucleate stage of development. The percentage of uninucleate pollens (Fig.29) reached to 60% in the third floral bud. Further in the fourth floral bud the percentage of uninucleate pollens decreased to 40% and it was 10% in the fifth bud (Table XX), mostly the pollens were in their binucleate or late binucleate stage of development.

Thus third, fourth and fifth buds in serial order of development containing uninucleate to binucleate pollens were used for further experiment.

b) Suitable stage of development of pollens for haploid induction

As from the result of the previous experiment, healthy third to fifth floral buds in serial order of development were collected and surface sterilized according to the procedures described in Chapeter II Materials and Methods. The anthers were inoculated into tubes and incubated at 25 ± 2°C for 16h photoperiod (1000 lux).

Results depicted in Fig.30 indicate that the cultured anthers of the third floral bud were most responsive (60%) as these anthers started swelling within ten days and by the end of three weeks the anther wall ruptured longitudinally (Fig.31). The rate of response decreased to 12% in the fourth bud, which was further reduced to 5% when the anthers of the fifth bud were tested (Fig.30).

Thus, the third floral bud in serial order of development containing anthers with mostly uninucleate pollens were found to be suitable for induction of haploids.

Fig. 29 Uninucleate pollens (400 X)

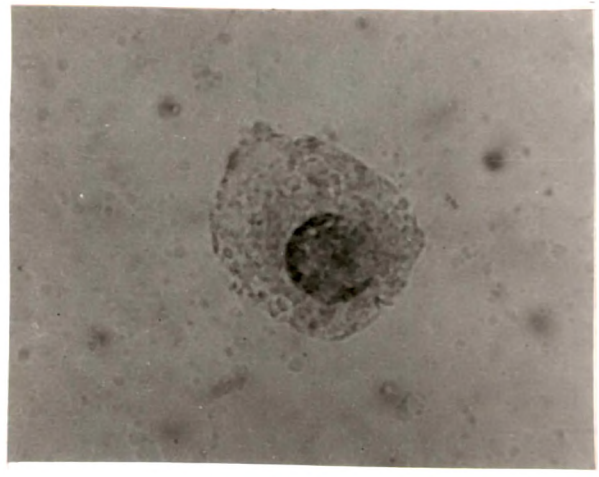


Fig. 29

Table XX : Selection of suitable bud for induction of haploids

Sr. No.	Buds in serial order of development from apex, within a cyme	Stage of pollen development	% uninucleate pollen
1.	First	PMC	-
2.	Second	Tetrad	5
3.	Third	Uninucleate	60
4.	Fourth	Binucleate	40
5.	Fifth	Late binucleate	10

Fig. 30 Response of cultured anthers of the third / fourth / fifth buds in serial order of development

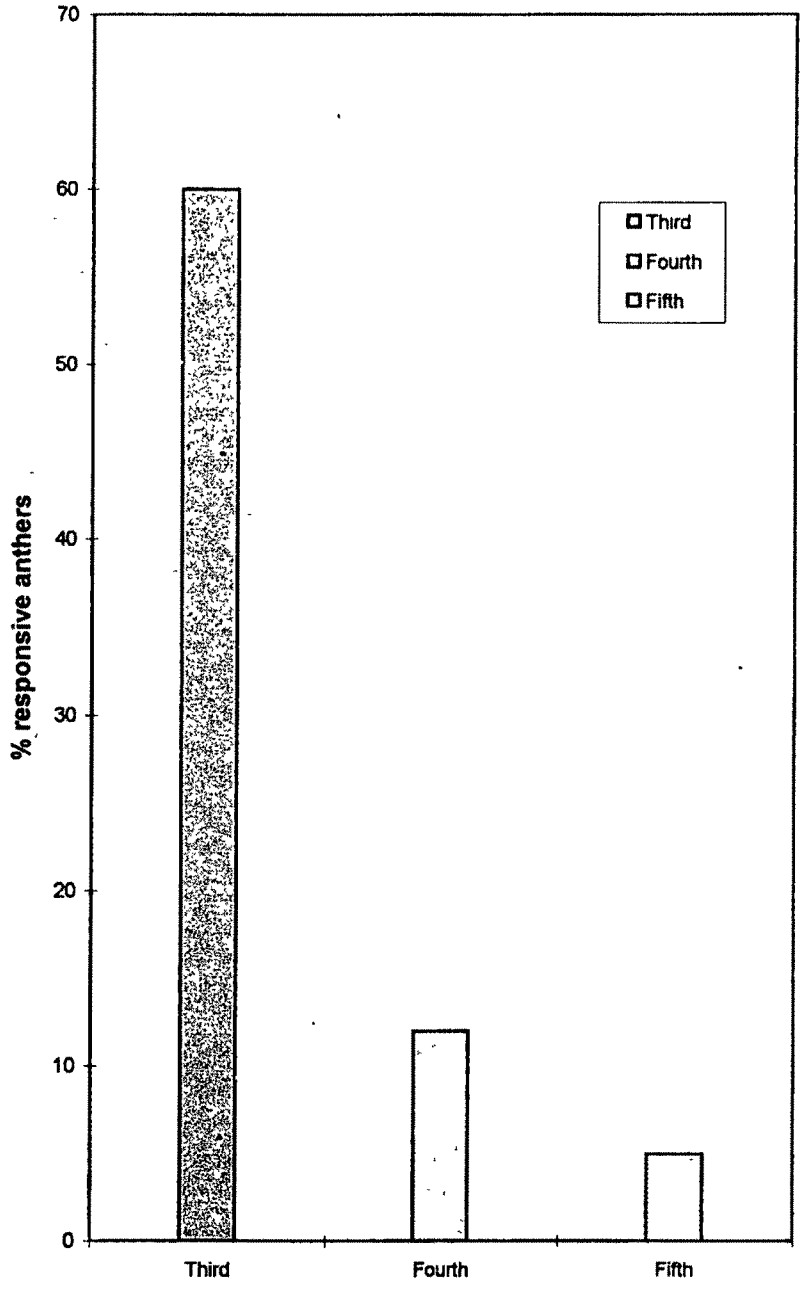

70
60
50
40
30
20
10
0
% responsive anthers
Third
Fourth
Fifth
Third
Fourth
Fifth
Buds in serial order of development

Fig. 30

Fig. 31 Responding anthers from the third floral bud on M S basal medium

Fig. 31

Experiment 24 : Effect of coconut water on anther/pollen culture

MS media (20 ml) with sucrose (2%) supplemented with various concentrations of coconut water (CW) (5, 10, 15, 20, 25%) were inoculated with anthers containing uninucleate pollens. Culture tubes were incubated according to procedures described in Chapter II Materials and Methods.

Results recorded in Table 25 indicate that at lower concentrations of CW (5, 10%) the morphogenic response observed was callusing of the anthers (70%). Microscopic observations revealed that the callus was initiated from the connective portion (Fig.32a) and it entirely covered the anthers within four weeks (Fig.32b). At higher concentrations of CW (15-20%), the anthers swelled (50%) and burst within two weeks and small embryoids were seen to emerge (Fig.33a). These were transferred to MS medium containing sucrose 4% and 15% CW which supported the growth of the embryoids which germinated into delicate plantlets at the end of four weeks (Fig.33b). Further increase in CW to 25% did not elicit any morphogenic response.

Thus, MS medium supplemented with 15% CW induced haploid plantlet from the pollens.

Experiment 25 : Production of homozygous diploids

In vitro haploid plants were raised from pollen culture were used to produce homozygous diploids. Axillary shoot buds of haploid plants were treated with cotton swab dipped in colchicine of different concentrations of 0.1, 0.5 and 1% for about 24, 48 and 72 hours. These buds were washed with sterile

Table XXI : Effect of conconut milk on anther/pollen culture

Medium : M S + sucrose (2%) + CM (5, 10, 15, 20, 25%)

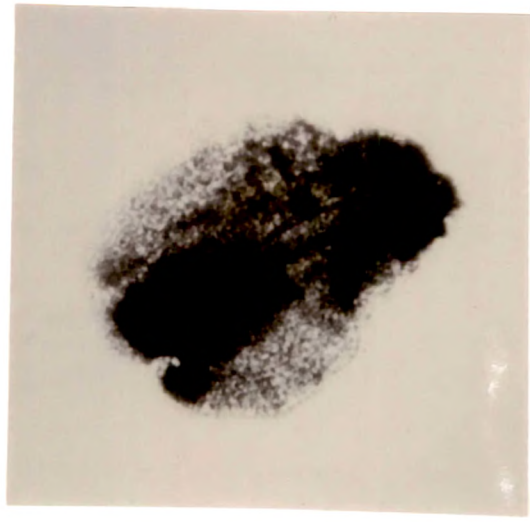
Incubation : 4 weeks in culture room at 25 ± 2°C for 16 h photoperiod (1000 lux)

Sr. No.	Coconut Milk %	Morphogenic response	% response
1	5	callusing	70
2	10	callusing	60
3	15	embryoid	50
4	20	embryoid	50
5	25	-	-

- No response

Fig. 32 (a) Callus formation from the connective of the anther lobe (160 X)

(b) Callus after four weeks of culture period



a

b

Fig. 32

Fig. 33 (a) Small embryoid formation after two weeks

(b) A delicate plantlet obtained after four weeks

a

b

Fig. 33

distilled water and were transferred to root-inducing medium. Within four weeks, healthy plantlets were developed in all the treated axillary buds. Out of these 0.5% colchicine treatment given for 48 hours in all the five replicates of plantlets were found to be healthy and normal (Fig.34a). Root tip of one of the plants when squashed showed the chromosome number to be 24 (Fig.34b). This indicated that the diploidisation of these haploid plants had occurred, as the chromosome number '2n' in *W. somnifera* is 24.

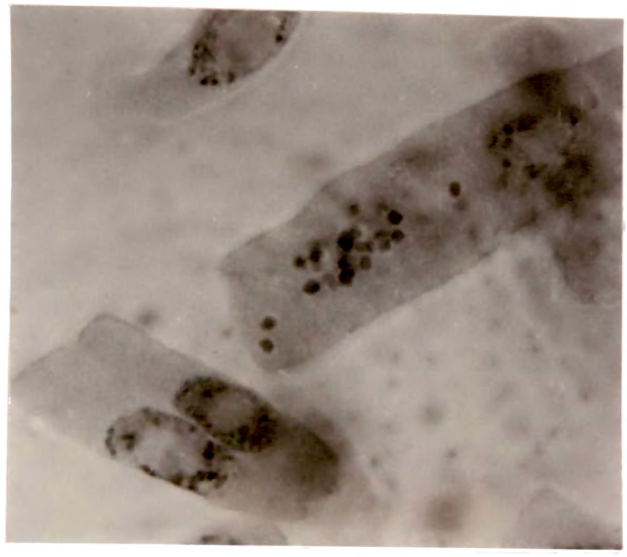
Plants were transferred to pots containing vermiculite and acclimatized to natural environmental conditions.

— x —

Fig. 34 (a) A diploid plantlet after eight weeks

(b) A metaphase plate from root tip of regenerated plant showing diploid chromosome number 2n = 24 (250 X)

a



b

Fig. 34