RESULTS

PART I

Boerhaavia diffusá L.

RESULTS

Boerhaavia diffusa L. is a prostrate perennial herb. The extract of this plant forms the source of drug namely punarnava. Its medicinal importance is due to the alkaloids namely punarnavine and two other unidentified ones which are synthesized and accumulated by the plant. Moreover, punarnava constitutes one of the ingredients in the various Ayurvedic medicines available.

This section of the thesis deals with the experimental work conducted on screening of *B. diffusa* L. plants for the presence of punarnavine alkaloid. Procedures for the extraction of the alkaloid, its isolation in pure form and identification were standardized. The quantification of punarnavine was based on dry weight. Also the site of accumulation of the alkaloid in the individual organ of the plant was determined.

SECTION A : *IN VIVO* SCREENING OF PLANTS

Experiment 1: Extraction, isolation and identification of Punarnavine alkaloid from *Boerhaavia diffusa* L. plants

Mature healthy *B. diffusa* L. plants in the M.S.University Campus were collected, washed under tap water, rinsed in distilled water and dried at room temperature. Also a Herbarium sheet of the plant was prepared, identified and maintained in the Departmental Herbarium (Fig.1). Each dried plant material was powdered. The powder (20 g) was extracted using ethanol as the solvent. The extraction was done according to the procedures described by Singh and Udupa

Fig.1 *Boerhaavia diffusa* L. Plant

Fig. 1

(1972) as given in Chapter II, Materials and Methods.

The extract was subjected to preparatory TLC, plates were developed and sprayed with Dragendorff's reagent. The spots turned to orange colour which confirmed the presence of punarnavine alkaloid. These spots were eluted, dried and redissolved in ethanol. In duplicate 2 ml aliquot of it was used for TLC along with the authentic sample of punarnavine. The Rf values (0.85) of the sample as well as punarnavine coincided (Fig.2) which confirmed that the isolated compound was `punarnavine' alkaloid.

Experiment 2: Punarnavine profile of the different organs of a *Boerhaavia diffusa* L. plant

A mature *B. diffusa* L. plant from the M.S.University Campus was collected, washed and its individual organs *viz.*, roots, stem, leaves and flowers were separately dried and were powdered. Punarnavine was extracted from 0.2 g of each sample according to Huber's (1967) method described in Chapter II, Materials and Methods.

Results recorded in Table III, showed that the quantity (dry weight basis) of punarnavine accumulated in individual organs of a single plant varied, highest being in roots (2%). Leaves accumulated (0.82%), flowers (0.65%) and lowest quantity was recorded in the stem portion of the plant (0.56%). The roots of this plant seem to be the site of accumulation of punarnavine.

TABLE III : Punarnavine contents in different organs of a *Boerhaavia diffusa* L. plant

Sr. No.	Plant Organ	Punarnavine %
1.	Roots	2.0
2.	Stem	0.56
3.	Leaves	0.82
4.	Flowers	0.65

% on dry weight basis

Fig.2 TLC of Punarnavine (a) authentic (b,c) isolated

Fig. 2

Experiment 3 : Alkaloid profile of roots of *Boerhaavia diffusa* L. plants

Well developed roots of *B. diffusa* L. plants, four to five years of age, growing in the M.S.University Campus and industrial areas such as Nandesari and G.I.D.C. Makarpura, Baroda were collected. Roots of the plants from the M.S.University Campus were thick (Fig. 3 a) while those of the other two places *viz.*, from Nandesari and Makarpura, were thin (Figs. 3 b, c) inspite of the same age of all these plants.

These root samples after washing and drying were powdered and punarnavine content was extracted following the procedure described by Huber (1967) given in Materials and Methods.

Results recorded in Table IV indicate that highest alkaloid content (2%) was in roots isolated from the plants growing in the M.S.University Campus. *B. diffusa* plants from the industrialized localities of Nandesari and Makarpura accumulated comparatively lower quantities of alkaloids in their roots (0.88% and 0.93%). Based on these results, plants growing in the M.S.University Campus proved `elite' superior amongst them. Hence, for experimental work these `elite' plants or their organs were used.

SECTION B : EXCISED ROOT CULTURES

Since the active principal of medicinal importance `punarnavine' was accumulated in roots of *B. diffusa* L. (Experiment 2), the excised root cultures of this plant were established in axenic cultures (Section : B). Studies regarding the

TABLE IV : Alkaloid contents in root samples of plants growing in different localities

Sr. No.	Localities	Punarnavine %
1.	M.S.University Campus	2.0
2.	Nandesari	0.88
3.	G.I.D.C. Makarpura	0.93

% on dry weight basis.

Fig.3 Roots of *Boerhaavia diffusa* L. collected from various localities (a) M.S.University Campus (b) Nandesari Industrial Area (c) G.I.D.C Makarpura Area

a

b

c

Fig. 3

nutritional/hormonal requirements of excised cultured roots were investigated. A medium which supported the highest biomass production, in terms of fresh and dry weights as well as the length of main axis, the number of laterals and alkaloid synthesis/accumulation was standardized. Root clones growing in aseptic stock cultures in liquid medium were developed for conducting the experimental work. In addition an attempt was made to enhance the punarnavine contents of cultured roots by feeding them with amino acid.

Experiment 4 : Selection of suitable culture medium for establishment of excised root cultures of *B. diffusa* L.

White's (1954) medium (W) specially formulated for the continuous growth of excised root cultures of angiosperms and Murashige and Skoog's (1962) medium (MS) were used in the present studies for culturing excised roots of *B. diffusa* L. Excised roots 1-2 cm in length with their tip intact, were isolated from aseptically germinated seeds of *B. diffusa* and were inoculated in Erlenmeyer flasks (150 ml) containing W or MS (40 ml) of liquid media with (2%) sucrose at pH 5.7. The culture flasks were maintained on gyratory shaker (120 rpm) in culture room at 25 $\pm$ 2°C, keeping six replicates for each treatment.

Results showed that the length of the main axis of root reached to 4.5 cm in MS medium in all the replicates (100%) with four lateral roots (Fig. 4 a). While in W medium roots showed poor growth, 3.1 cm in length without any lateral roots produced (Fig. 4 b). The superiority of MS medium was thus proved over that of W medium as it supported active growth of excised roots of *B. diffusa* L. in sterile

Fig.4 Excised roots of *Boerhaavia diffusa* after culturing for four weeks in (a) MS medium (b) W medium

a

Fig. 4 b

culture. Based on this result, MS medium was selected as root culture medium for the excised roots of this plant.

Experiment 5: Effect of phytohormones *viz.*, cytokinin - Kn/BAP alone and Kn in combination with auxin - IAA/IBA/NAA/2,4-D - on growth of excised roots in culture

(i) Effect of Kn :

Excised roots with tips of germinated seeds of `elite' *B. diffusa* were inoculated in MS (40 ml) medium containing sucrose (2%) and supplemented with Kn at 0.5 or 1.0 or 2.0 μM/l levels. Results after four weeks incubation in culture room (25 $\pm$ 2°C) on gyratory shaker, showed that Kn at 0.5 μM/l supported active growth of cultured root as the main axis was 6.5 cm in length with the production of 6 $\pm$ 1 lateral roots (Fig.5). Further increase in Kn level to 1 μM/1, led to linear increase in the length of main axis to 10.1 cm with production of lateral root to 9 $\pm$ 1. However, at 2 μM/l of Kn, these growth parameters of cultured roots were reduced when compared to with those resulted at 1 μM/l of Kn. Thus,Kn at 1 μM/l proved to be the optimal level for culturing excised roots of *B. diffusa* L.

(ii) Effect of BAP :

With the change of cytokinin to BAP, there was reduction in all growth parameters of excised roots when compared with those obtained with Kn concentrations (Fig.5). At 0.5 μM/l of BAP, the length of the main axis was 5 cm with a single lateral root produced. Even at 1μM/l of BAP, the main axis of root

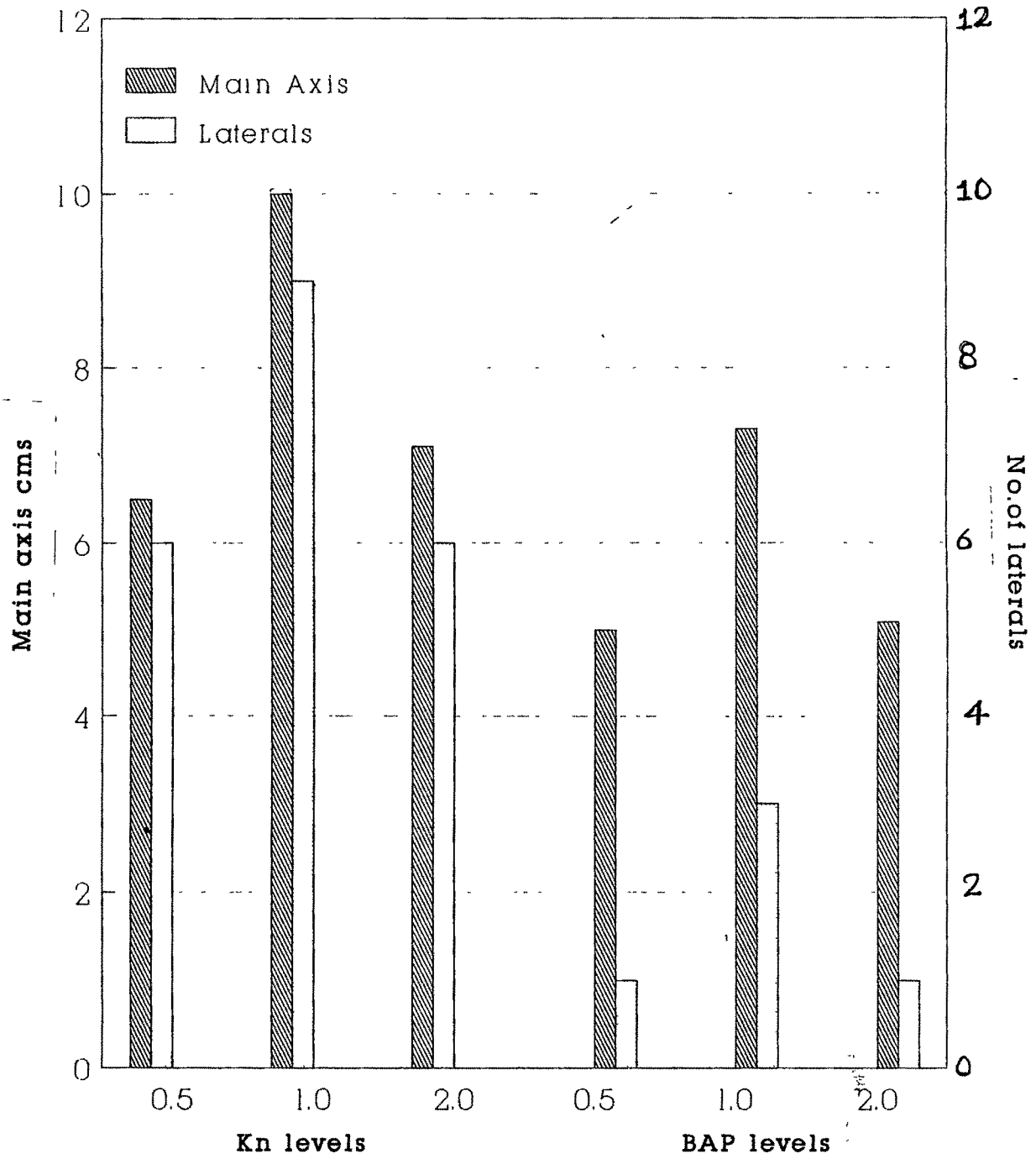


Fig.5 Effect of various levels of cytokinin (Kn/BAP) on excised root culture

recorded 7 ± 2 cm of length with the production of 3 ± 1 lateral roots. Same was true with 2 μM/l of BAP treatment.

Out of the two cytokinins, Kn proved superior over BAP because it supported not only the highest growth of the main axis of the root but it also produced the highest number of lateral roots. Hence, based on this result, Kn 1 μM/l was incorporated in root culture medium of *B. diffusa* L. for further experimental work.

(iii) Effect of IAA in combination with Kn (1 μM/l) :

The culture medium was incorporated with IAA at levels of 0.5, 1.0, 2.0 and 4.0 μM/l and the excised roots with their tips intact were grown in them for four weeks as per standard procedure. Results recorded in Table V show that the main root length was 7.5 cm along with 6 ± 1 lateral roots at 0.5 μM/l of IAA when combined with 1 μM/l of Kn. In fact, 2μM/l of IAA supported active growth of main axis which reached to 13.7 ± 0.1 cm in length with 10 ± 2 lateral roots (Fig. 6 a). However, 4 μM/l of IAA incorporation in the culture medium, retarded the growth of the main axis of root from 13.7 cm to 8.2 cm in length and the number of lateral roots from 10 to 7 ± 1.

(iv) Effect of IBA in combination with Kn (1 μM/l)

Culture medium of roots was supplemented with IBA at 0.5, 1, 2, 4 μM/l in combination with Kn 1 μM/l. Results indicate that the length of main axis of root was 9.5 ± 0.1 cm with 8 ± 1 lateral roots produced in response to 0.5 μM/l IBA incorporated in the culture medium. Highest length in the main axis was 17.1 ± 0.12 cm with 22 ± 2 lateral roots at 2 μM/l of IBA treatment (Fig. 6 b). Further

Table V : Effect of auxin in combination with Kn (1 μM/l) on the growth of cultured roots

Medium : MS + sucrose (2%) + Kn (1μM/l) + IAA/IBA/NAA/2,4-D (0.5,1,2,4 μM/l)

Inoculum : Root with its tip (1-2 cm)

Incubation: 4 weeks in culture room on gyratory shaker (120 rpm) at 25 ± 2°C

Sr. No.	Auxin levels μM/l	Length of main root axis (cm)	No.of lateral roots	Response %
IAA				
1	0.5	7.5 ± 0.06	6 ± 1	80
2	1.0	12.3 ± 0.01	10 ± 2	70
3	2.0	13.7 ± 0.1	10 ± 2	100
4	4.0	8.2 ± 0.04	7 ± 1	60
IBA				
5	0.5	9.5 ± 0.1	8 ± 1	80
6	1.0	15.2 ± 0.07	12 ± 2	60
7	2.0	17.1 ± 0.12	22 ± 2	100
8	4.0	10.5 ± 0.1	12 ± 2	60
NAA				
9	0.5	5.0 ± 0.1	3 ± 1	60
10	1.0	6.0 ± 0.2	4 ± 1	80
11	2.0	6.6 ± 0.1	5 ± 1	60
12	4.0	5.1 ± 0.2	4 ± 1	60
2,4-D				
13	0.5	-	-	60
14	1.0	C	-	100
15	2.0	C	-	100
16	4.0	C	-	70

Mean of six replicates with standard deviation
C - callus
- Nil

increase in IBA level to 4 μM/l reduced these growth parameters (10.5 $\pm$ 0.1 cm in length with 12 $\pm$ 2 lateral roots). This data indicate superiority of 2 μM/l IBA treatment over other treatments.

(v) Effect of NAA in combination with Kn (1 μM/l)

The third auxin tested was NAA in combination with Kn (1 μM/l). At 0.5 μM/l of NAA, the main axis recorded was 5 $\pm$ 0.1 cm in length with 3 $\pm$ 1 lateral roots. The higher levels of NAA *viz.*, 1 or 2 μM/l resulted in slight increase in the main axis length, 6 $\pm$ 0.2 cm and 6.6 $\pm$ 0.1 cm respectively with 4 $\pm$ 1 and 5 $\pm$ 1 lateral roots (Fig. 6 c). Further 4 μM/l of NAA, the main axis reached to only 5.1 $\pm$ 0.2 cm with 4 $\pm$ 1 laterals which means that there was a decline in the growth of the main axis as well as in the number of laterals. When these results of NAA treatments were compared with those of the IBA treatment, the increase in the main axis length of the root and the number of lateral roots developed was highest in response to IBA versus NAA treatments.

(vi) Effect of 2,4-D with Kn (1 μM/l)

In excised roots grown in 2, 4-D supplemented medium (2 and 4 μM/l) with Kn (1 μM/l), callus was induced at the root tips within one week culture period. After a week, induction of callus occurred from root tips in 1 μM/l of 2,4-D supplemented medium (Table V). Callus was cream coloured which completely covered the explant at the end of four weeks culture period. However, no callus was induced from cultured roots in lower level (0.5 μM/l) of 2, 4-D supplemented medium.

Fig.6 Excised root cultured in MS medium supplemented with sucrose (2%) + Kn (1 μM/l) + auxin (a) IAA (b) IBA (c) NAA (2 μM/l)

Incubation : Four weeks in light at 25 $\pm$ 2°C

a

Fig. 6

b

Fig. 6

c

Fig. 6

Results of this experiment proved that root-clones of excised roots of *B. diffusa* L. could be produced when MS medium was supplemented with Kn 1 μM/l and IBA at 2 μM/l. Hence, the medium supplemented with these two phytohormones was selected as the root culture medium.

Experiment 6 : Determination of optimal sucrose level for continuous growth of excised roots in culture

Sucrose in the culture medium being the sole energy source available to the cultured roots, its optimal level supporting the highest growth in the length of main axis of root with number of lateral roots was determined. Excised roots with their tips from germinated seeds were inoculated in MS (40 ml) medium containing Kn (1 μM/l), IBA (2 μM/l) and supplemented with sucrose at various levels.0, 1, 2, 3, 4%. Culture flasks were maintained on gyratory shaker in culture room at 25 $\pm$ 2°C, 16 h light (1000 Lux).

After four weeks of culture period, cultured roots turned black and died in absence of sucrose. This clearly proved that for *in vitro* root cultures, an energy source such as sucrose was essential. Addition of sucrose at 1%, stimulated the growth of main axis as well as initiation of lateral roots (Fig. 7). At 2% sucrose, length of the main axis was the highest (17.1 $\pm$ 0.12 cm) with the highest number of lateral roots (22 $\pm$ 2). Further, increase in sucrose level to 3% or 4% not only reduced the growth of the main axis of root but also the number of lateral roots produced which confirmed that sucrose at 2% was the optimal level required for the continuous culture of excised roots of *B. diffusa* L.

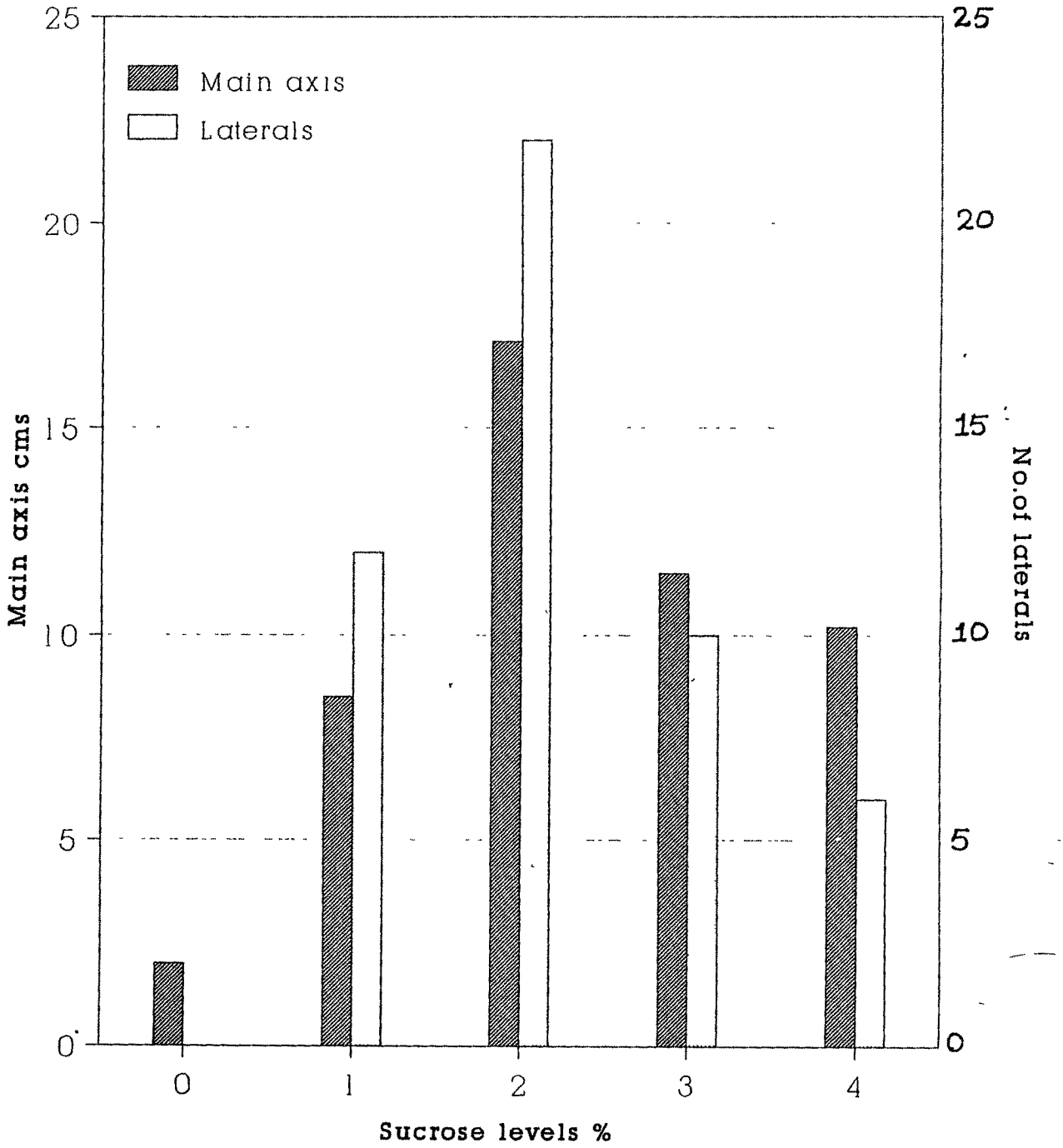


Fig.7 Growth of excised roots at various concentrations of sucrose after four weeks

Experiment 7 : Effect of variation in concentrations of salts (half, standard, double) of MS medium on growth of excised roots in culture

The present experiment was conducted to investigate the effect of the variation in the concentrations of salts of MS medium to one half concentration, standard dose and double the concentrations, supplemented with sucrose (2%), Kn (1 μM/l) and IBA (2 μM/l) on the growth of the excised roots in culture. Culture flasks (250 ml) containing media (120 ml) were inoculated with ten roots (1-2 cm) and were incubated on gyratory shaker in culture room at 25 $\pm$ 2°C.

Results revealed that the highest growth in terms of fresh weight (790 $\pm$ 15.7 mg) and dry weight (55 $\pm$ 1.5 mg) occurred when MS medium salts were present at the standard dose (Fig. 8 a). Also, doubling the concentrations of salts of MS medium, the growth parameters of cultured roots were reduced in terms of fresh weight (348 $\pm$ 11.3 mg) and dry weight (21 $\pm$ 0.9 mg) at the end of four weeks period (Fig 8 b). Reducing the concentrations of salts to half of MS medium also drastically reduced the fresh and dry weights of cultured excised roots (Fig. 8 c).

The salt concentration as present in the MS standard medium was found most ideal (Table VI) since it supported the highest growth in terms of fresh and dry weights of cultured roots of *B. diffusa* L.

Table VI : Effect of half, standard and double concentration of salts of MS medium on growth of excised roots in culture

Medium : MS (half/standard/double) + sucrose (2%) + Kn (1μM/l) + IBA (2 μM/l)

Inoculum : 10 roots with tips (1-2 cm)
Fresh weight = 7 mg
Dry weight = 0.3 mg

Incubation: 4 weeks in culture room on gyratory shaker (120 rpm) at 25 $\pm$ 2°C

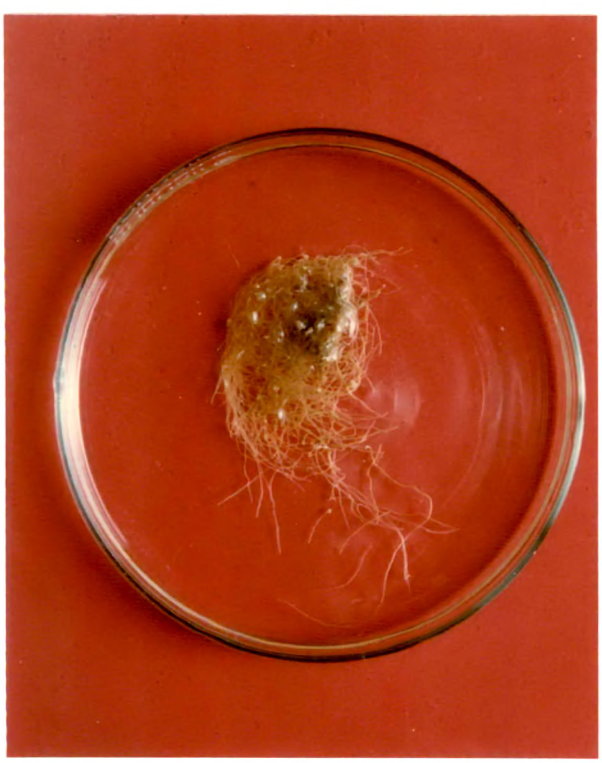
Sr. No.	Salt Concentration in MS medium	Fresh weight mg	Dry weight mg
1	Half	215 $\pm$ 7.2	12.8 $\pm$ 0.5
2	Standard	790 $\pm$ 15.7	55.0 $\pm$ 1.5
3	Double	348 $\pm$ 11.3	21.0 $\pm$ 0.9

Mean of six replicates with standard deviation

Fig.8 Excised roots cultured in MS medium of salts (a) Standard dose (b) double (c) half, with sucrose (2%), Kn (1 μM/l) and IBA (2 μM/l)

Incubation : Four weeks in light at 25 $\pm$ 2°C

a



b

c

Fig. 8

Experiment 8 : Effect of variation in vitamin levels in MS medium on growth of excised roots in culture

Vitamins form one of the necessary ingredients of the culture medium, even though they are added in minute quantities, they exert profound effect particularly on cultured roots. The present experiment was carried out to find the effect of vitamins on the growth of excised root cultures of *B. diffusa* L. Excised roots (1-2 cm) were cultured in 40 ml of MS medium excluding the vitamins, second, in MS medium with standard dose of vitamins, third, reducing the level of vitamins to one half and in fourth, doubling the level of vitamins, supplemented with sucrose (2%), Kn (1 μM/l) and IBA (2 μM/l). Incubation of cultures was done as per the standard procedures.

Results recorded (Table VII) after four weeks clearly indicate that growth of excised roots was poor in absence of vitamins while in vitamins at their one-half level the growth was improved (Fig.9 a). That proved the requirement of vitamins for the normal growth of cultured roots. At standard dose of vitamins as present in the MS medium, all the growth parameters of roots, namely the length of the main axis (17.1 $\pm$ 1 cm) and the number of lateral roots produced (25 $\pm$ 2) were the highest (Fig. 9 b). Increasing the vitamin level from its original level to double, a reduction in the main axis length (14.6 $\pm$ 0.5 cm) along with the number of lateral roots (17 $\pm$ 2 cm) was observed. These findings indicate that vitamins were essential for the growth of excised roots in culture and their dose as present in MS standard medium was optimal for *B. diffusa* L. root cultures.

Table VII : Effect of variation in vitamin levels on growth of excised roots in culture

Medium : MS without vitamins + sucrose (2%) + Kn (1μM/l) + IBA (2 μM/l) + various levels (absence, half, standard,double) of vitamins of MS medium

Inoculum : Root with tip (1-2 cm)

Incubation: 4 weeks in culture room on gyratory shaker (120 rpm) at 25 ± 2°C

Sr. No.	Levels of Vitamins	Length of main axis cm	No.of laterals
1	Absent	5.2 ± 0.09	3 ± 1
2	Half	9.2 ± 0.10	3 ± 1
3	Standard	17.1 ± 1	25 ± 2
4	Double	14.6 ± 0.5	17 ± 2

Mean of six replicates with standard deviation

Fig.9 Excised root cultured in MS medium with (a) half dose of vitamins (b) standard dose of vitamins, after four weeks

a

b

Fig. 9

Experiment 9 : Growth kinetics of excised roots in culture

The progress in growth of excised cultured roots in standard MS medium (40 ml) was determined in terms of the length of main axis and the number of lateral roots produced at weekly intervals for a five weeks period. Five of the replicate flasks were harvested after each week and the length of the main axis and the number of lateral roots produced were recorded according to the procedures described in Materials and Methods, Chapter II.

The graph of growth parameters plotted against time showed the typical sigmoid curve (Fig. 10). The rate of growth of the main root axis and the number of lateral root formation was rapid during first three weeks period, reaching to its maximum (17.2 $\pm$ 1.0 cm length and 25 $\pm$ 2 lateral roots) at the end of fourth week. During fifth week period, both these growth parameters declined.

Experiment 10 : Punarnavine profile of cultured roots of *Boerhaavia diffusa* L.

The present experiment was undertaken to find out whether excised cultured roots could synthesize/accumulate punarnavine alkaloid which was isolated from the roots of *B. diffusa* plants growing *in vivo*. About ten roots (1-2 cm long) with their tips were excised from germinating seeds and grown in MS medium (120 ml) containing sucrose 2%, Kn (1 μM/l) and IBA/IAA/NAA (2 μM/l). The growth parameters of excised roots cultured in medium supplemented with sucrose (2%), Kn(1 μM/l) along with IBA (2 μM/l) recorded the highest values as was observed in the results of Experiment 5 and hence, 2 μM/l level of auxin was selected for the present experiment. Culture flasks were incubated on gyratory shaker in culture

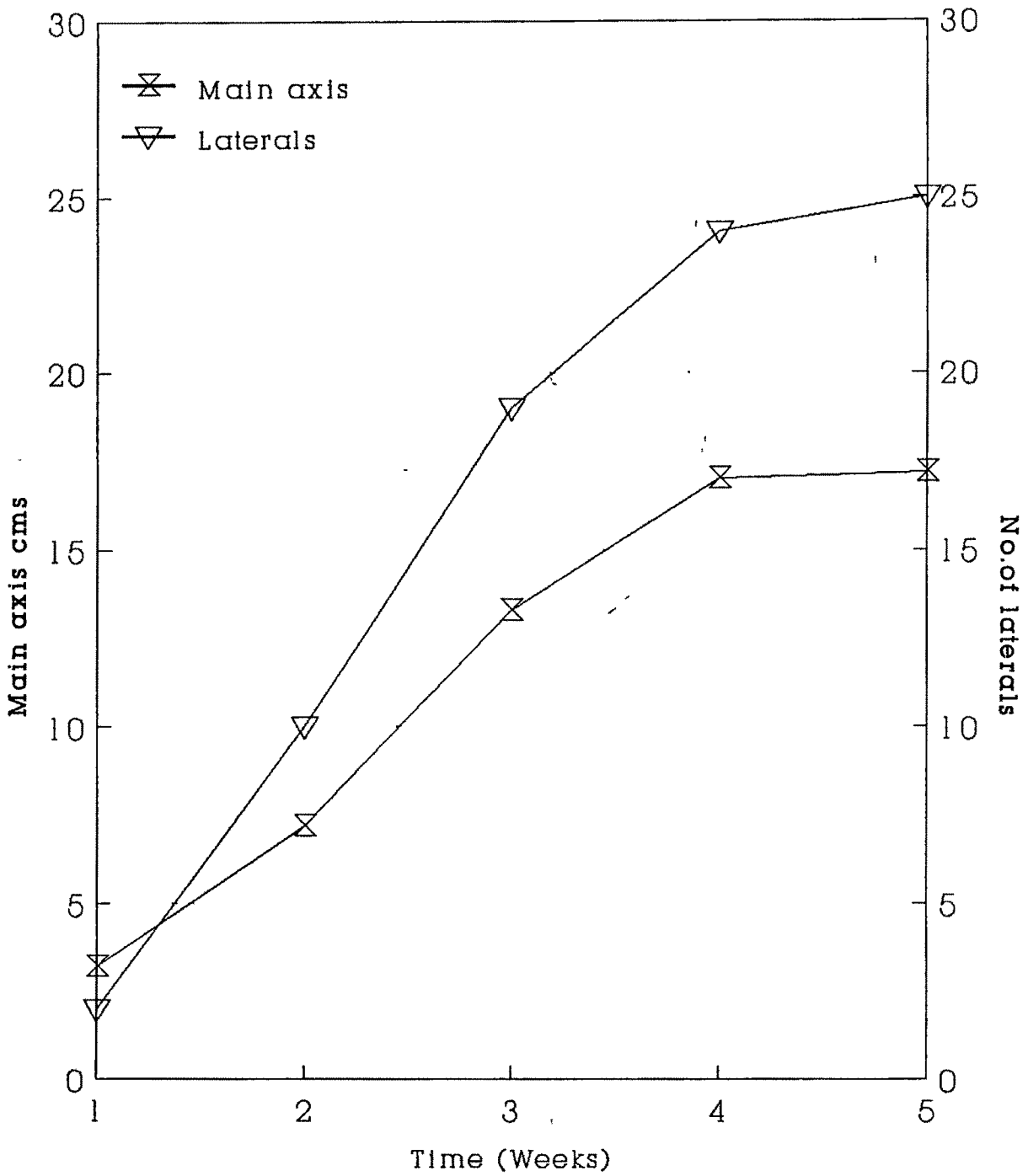


Fig.10 Growth pattern of *in vitro* excised roots

room at 25 ± 2°C.

Results of this experiment (Table VIII) recorded after four weeks culture period showed that out of three auxin treatments, IBA at 2 μM/l supported highest biomass production of cultured roots and their fresh and dry weights were 796 ± 11.6 mg and 55 ± 3.1 mg respectively. Punarnavine contents estimated 0.008% calculated on dry weight basis.

Roots cultured in IAA 2 μM/l supplemented medium recorded 535 ± 6.5 mg fresh weight and 27.9 ± 2 mg of dry weight. However, the punarnavine content was negligible as only its presence was detected.

Results in response to NAA 2 μM/l treatment recorded lowest biomass values *viz.*, 265 ± 4.5 mg and 13.6 ± 0.6 mg fresh and dry weights respectively. However, presence of punarnavine contents could not be detected (Table VIII).

Experiment was continued further by adding fresh medium (120 ml) of the same composition to each of the cultured roots and the culture flasks were maintained on gyratory shaker in culture room.

Results recorded after eight weeks (Table VIII) showed that there was not only enhancement in the biomass production of cultured roots but also it was in their punarnavine contents.

Roots in response to IBA treatment (Fig. 11 a) recorded the highest biomass *viz.*, 1279 ± 25 mg fresh weight and 78 ± 1.6 mg dry weight values (Table VIII)

Table VIII : Punarnavine profile of cultured roots of *Boerhaavia diffusa* L.

Medium : MS + sucrose (2%) + Kn (1μM/l) + IAA/IBA/NAA (2 μM/l)

Inoculum : 10 roots with tips (1-2 cm)
Fresh weight - 7 mg
Dry weight - 0.3 mg

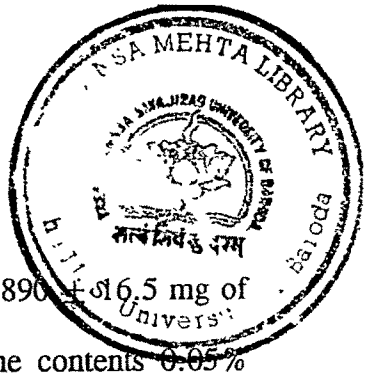
Incubation: 8 weeks in culture room on gyratory shaker (120 rpm) at 25 ± 2°C

Sr. No.	Auxins	Fresh weight mg	Dry weight mg	Punarnavine %
After 4 weeks				
1	IAA	535 ± 6.5	27.9 ± 2	PD
2	IBA	796 ± 11.6	55.0 ± 3.1	0.008
3	NAA	265 ± 4.5	13.6 ± 0.6	-
After 8 weeks				
4	IAA	890 ± 16.5	60 ± 4	0.05
5	IBA	1279 ± 25	78 ± 1.6	0.06
6	NAA	410 ± 10	24.1 ± 2	0.002

Mean of six replicates with standard deviation

PD : Presence Detected

- : Nil



as well as the punarnavine content (0.06%).

Roots with IAA treatment grew well (Fig. 11 b) and recorded 890 ± 16.5 mg of fresh weight and 60 ± 4 mg dry weight with their punarnavine contents 0.05% (Table VIII). Roots cultured in NAA treatment (Fig. 11 c) recorded not only lowest biomass values but their punarnavine contents was also lowest (0.002%).

These results indicate that after eight weeks of culture period, the highest punarnavine alkaloid (0.06%) was found in the cultured roots of IBA treatment.

Experiment 11 : Effect of feeding L-tryptophan to cultured roots on their punarnavine synthesis/accumulation

Tryptophan being the precursor of punarnavine, it was added to root culture medium to find out its effect on punarnavine synthesis/accumulation by the cultured roots. About ten roots with tips intact (1-2 cm) isolated from germinated seeds of *B. diffusa* L. were inoculated in MS standard medium (120 ml) supplemented with L-tryptophan at various levels (5, 10, 20, 30 μM/l). Culture flasks were maintained on gyratory shaker in culture room.

The biomass in terms of fresh and dry weights along with alkaloid contents of the cultured roots were estimated as per the procedures described by Huber (1967) and recorded (Table IX). After eight weeks incubation period of the cultures, feeding of L-tryptophan to excised roots enhanced the biomass production gradually with the punarnavine contents. The highest biomass in terms of fresh weight (1320 ± 35 mg) and of dry weight (101 ± 10.2) was recorded when tryptophan level was

Table IX : Effect of L-tryptophan on punarnavine contents of excised roots

Medium : MS + sucrose (2%) + Kn (1μM/l) + IBA (2 μM/l) + tryptophan (5,10,20,30 μM/l)

Inoculum : 10 roots with tips (1-2 cm)

Incubation: 8 weeks in culture room on gyratory shaker (120 rpm) at 25 ± 2°C

Sr. No.	Levels of tryptophan μM/l	Fresh weight mg	Dry weight mg	Punarnavine %
1	00	1201 ± 24	75.5 ± 9	0.06
2	05	1290 ± 30	85.0 ± 9	0.11
3	10	1300 ± 30	97.0 ± 9.5	0.18
4	20	1320 ± 35	101.0 ± 10.2	0.32
5	30	1000 ± 20	65.0 ± 5.0	0.22

Mean of six replicates with standard deviation

% on dry weight basis

20 μM/l of culture medium (Fig. 12). At this level, presence of highest contents of punarnavine alkaloid synthesized/accumulated in the cultured roots was observed (0.32%). However, further increase in tryptophan level not only reduced the biomass production of excised roots but also reduced their alkaloid contents (Table IX).

Experimental results clearly indicate that feeding of L-tryptophan to excised roots was beneficial for the production of punarnavine alkaloid.

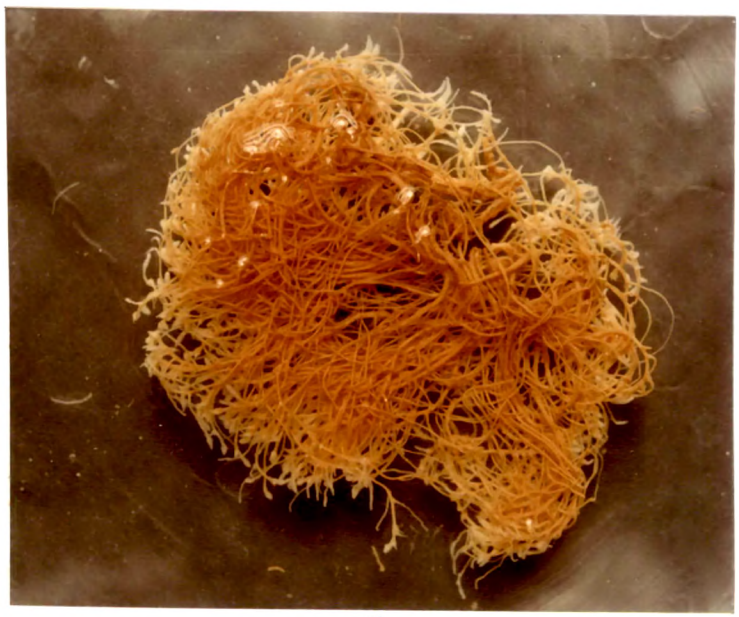
SECTION C : CALLUS CULTURES AND PLANTLET REGENERATION

Experiments described in this section were aimed at successful establishment of callus tissues from various organs of the `elite' superior *B. diffusa* L. Murashige and Skoog's (1962) medium containing inorganic salts, sucrose, supplemented with requisite dose of phytohormones was used for callus initiation.

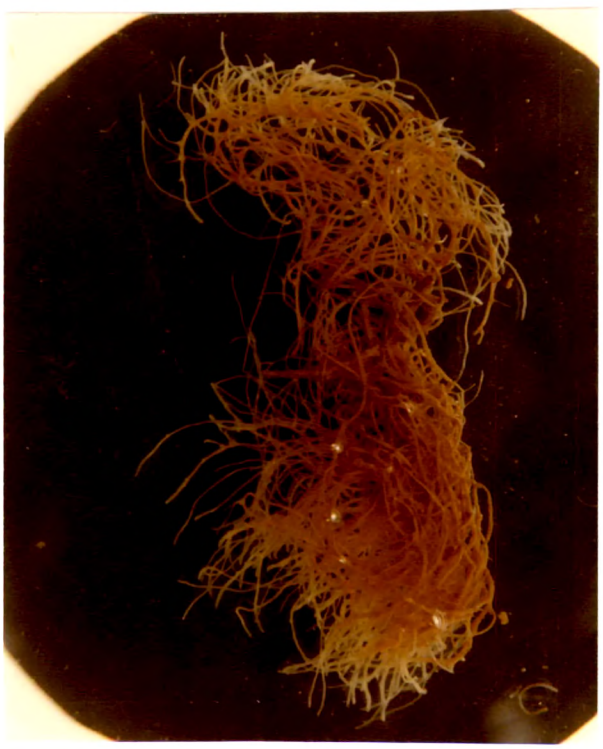
To carry out experimental work on callus, an adequate supply of callus was the basic requirement. Hence, experiments were conducted first to study the conditions promoting continuous growth of the callus in culture. Callus tissues originated from various organs were screened for punarnavine contents accumulated in them. Further an attempt was made to enhance the alkaloid content of callus masses. The morphogenetic and histogenetic pattern of callus differentiation was investigated during plant regeneration. Regenerated plants were successfully transferred to field conditions in order to raise uniform population of *B. diffusa* L.

Fig.11 Excised root cultured in MS standard medium with 2 μM/l of (a) IBA (b) IAA (c) NAA

Incubation : Eight weeks at 25 $\pm$ 2°C



a



b

c

Fig. 11

Fig. 12 Excised cultured roots in MS standard medium supplemented with L-tryptophan (20 μM/l)

Incubation : Eight weeks at 25 $\pm$ 2°C

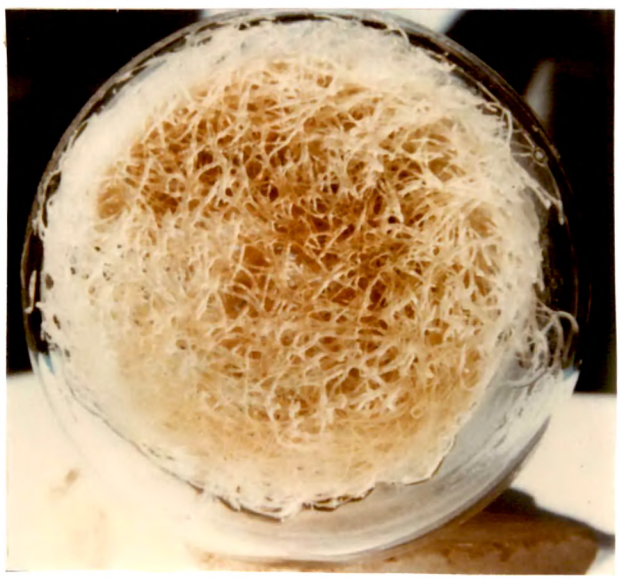


Fig. 12

Experiment 12 : Initiation of callus from stem, leaf, floral bud of `elite' *B.diffusa* L.

Healthy stem pieces, leaf and floral buds excised from *B. diffusa* L. plants after surface sterilization according to the procedures described in Chapter II, Materials and Methods, were inoculated on MS medium (30 ml) containing sucrose (2%) supplemented with Kn (0 to 4 μM/l) in combination with IAA/NAA/2,4-D (0 to 4 μM/l), pH 5.8 before gelling with agar (0.8%). Six replicates per treatment were maintained. Culture flasks were incubated in culture room at 25 $\pm$ 2°C in 16 h photoperiod (1000 lux) for four weeks culture period.

Table X shows no callus initiated from any of the cultured explants in absence of Kn and auxin. In Kn alone also no callus initiation occurred from these explants.

Callus was initiated from cut ends of stem explants when Kn levels were 0.5, 1 and 2 μM/l with IAA at 2 and 4 μM/l levels. Callus was cream coloured. In leaf explants, roots were regenerated in response to Kn and IAA treatments (Table X). From floral buds - calyx or corolla, no callus was induced even after four week culture period.

Replacing IAA with NAA, at all combination failed in callus initiation in any of the explants (Table X).

However, Kn in combination with 2,4-D supported maximum callus induction in stem and leaf explants at all the levels tested. Higher concentrations of Kn (1 and 2 μM/l) with 2,4-D induced callus much earlier than at it's lower concentrations.

Table X : Effect of Kn in combination with auxin (IAA/NAA/2,4-D) on callus induction from stem/leaf/floral bud explant of *Boerhaavia diffusa* L.

Sr. No.	Auxin µM/l	Kn	Stem					Leaf					Floral Bud				
			00	0.5	1.0	2.0	4.0	00	0.5	1.0	2.0	4.0	00	0.5	1.0	2.0	4.0
IAA																	
1	00		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2	0.5		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3	1.0		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4	2.0		-	+	+	-	-	-	R	R	R	-	-	-	-	-	-
5	4.0		-	++	+	-	-	-	R	R	R	-	-	-	-	-	-
NAA																	
6	0-4	No response															
2,4-D																	
7	00		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
8	0.5		-	+	+	+	-	-	++	++	+	-	-	++++	++++	++	-
9	1.0		-	+++	++	++	++	-	++++	++	+	+	-	+++	++	+	-
10	2.0		-	++++	+++	++	+	-	++++	++	++	+	-	++	+	++	-
11	4.0		-	+++	+	+	+	-	+++	++	+	+	-	+	+	++	-

Mean of six replicates

- No response , + denotes amount of callus induced, R roots

Also the amount of callus formed was more, at higher levels of 2,4-D than at lower levels (Fig.13 a, b). In floral buds neither calyx nor corolla induced callus.

Histological studies of callus induction in stem explants showed that the cortical parenchyma cells in response to 2,4-D treatment induced callus. During the process of callus formation the epidermal cell layer was disorganized (Fig. 14).

Results of this experiment proved that Kn and 2,4-D both were essential for callus induction from stem and leaf explants. Stem and leaf callus cultures were used as experimental material for further studies.

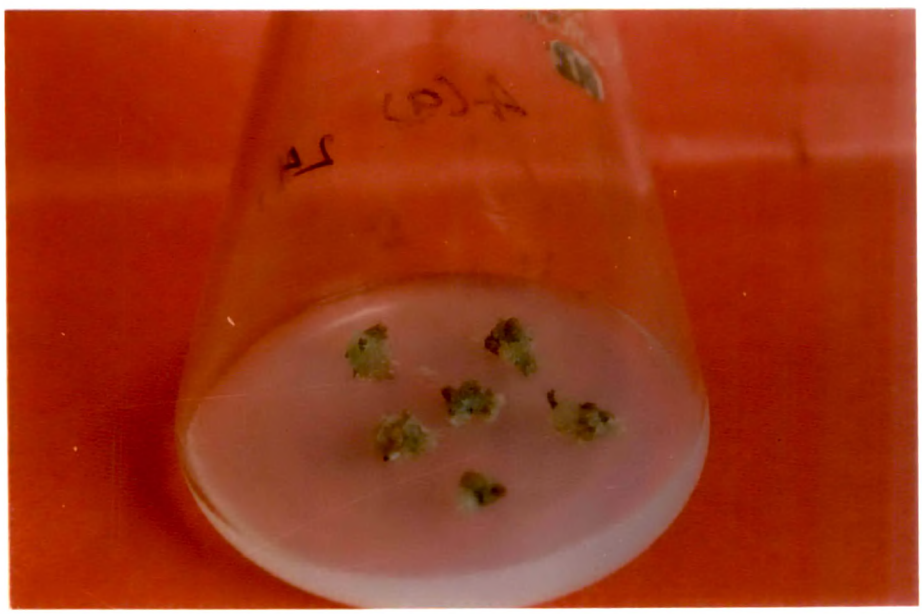
Experiment 13 : Biomass production of callus :

(i) Effect of Kn levels on biomass production :

Stem and leaf calli (300 ± 20 mg) were inoculated on MS medium (30 ml) containing sucrose (2%), 2,4-D (2 μM/l) supplemented with Kn (0 to 4 μM/l) and gelled with agar (0.8%).

After four weeks, a slight increase in biomass in terms of fresh and dry weights occurred in absence of Kn (Fig. 15 a) possibly due to its carry over effects of previous treatment. Addition of Kn at 0.5 μM/l induced callus growth and the values of fresh and dry weights recorded were respectively 962 ± 25 and 910 ± 22.2 mg and 48.10 ± 9 and 45.50 ± 7 mg of stem and leaf callus respectively. Further increase in Kn level to 1 μM/l enhanced the biomass production to its optimal level as seen in Fig 15 a. However, 2 and 4 μM/l of Kn levels in the

Fig.13 Callus induction in (a) Stem pieces (b) leaf pieces, after one week culture period



a

b

Fig. 13

Fig.14 T.S. of stem showing callus induction (c) from cortical parenchyma with the epidermal cell layer disorganised (160 X)

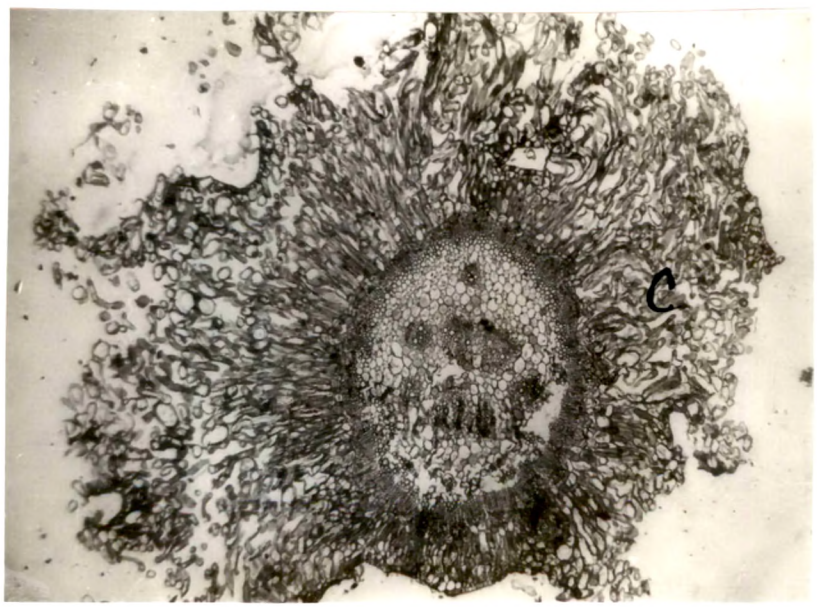


Fig. 14

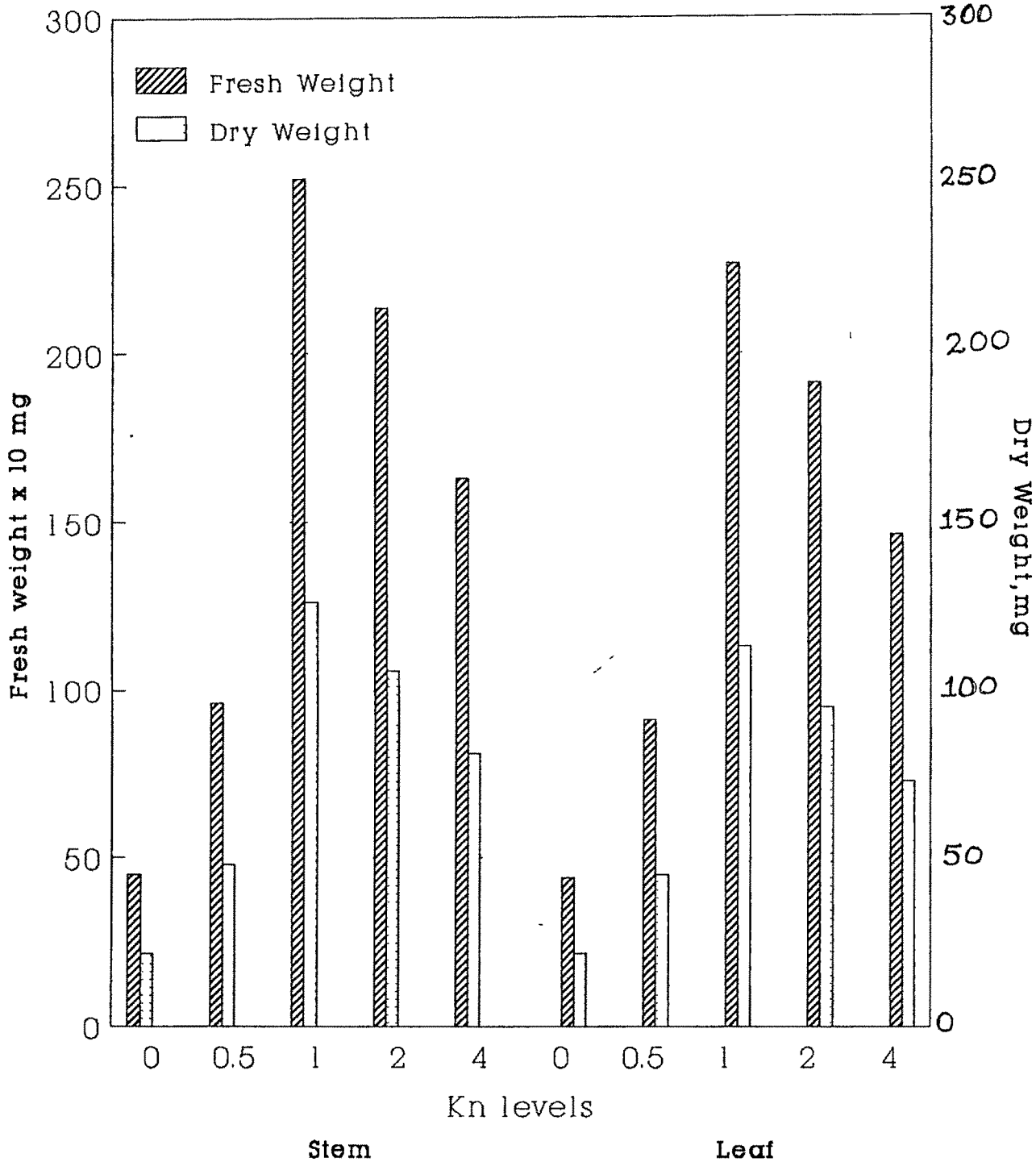


Fig. 15a Growth of callus tissues of stem and leaf at various concentrations of Kn

medium reduced the growth of callus whereby the production of biomass decreased.

It was concluded that Kn at 1 μM/l was the optimal level for callus growth.

(ii) Effect of 2,4-D levels on biomass production :

In this factoral experiment, the MS medium (30 ml) containing sucrose (2%) Kn (1 μM/l) supplemented with 2,4-D at various levels (0 - 10 μM/l) was inoculated with 300 $\pm$ 20 mg of stem and leaf callus tissues. Results depicted in Fig. 15 b show that in absence of 2,4-D, no growth in terms of fresh and dry weights of both these callus cultures occurred. With 2,4-D incorporation in the medium the growth of callus tissues resumed. Addition of 2, 4-D at 8 μM/l in the culture medium induced growth of the callus tissues to an optimal biomass production. On fresh weight basis the stem and leaf calli weighted 4449 $\pm$ 52 mg and 4105 $\pm$ 29 mg respectively. Their corresponding dry weights were 218 $\pm$ 20 mg and 205 $\pm$ 16 mg (Fig.16 a, b). It was also observed that the callus tissues were yellowish in colour and it turned to lighter colour with the passage of time.

This experiment proved Kn at 1 μM/l and 2, 4-D at 8 μM/l were at their optimal levels for maximum biomass production of both the callus tissues.

Experiment 14 : Effect of sucrose concentration on biomass production of callus tissues

The MS medium (30 ml) containing Kn (1 μM/l), 2, 4-D (8 μM/l) supplemented with sucrose at either 0, 1, 2, 3 or 4% was inoculated with 300 $\pm$ 20 mg of fresh stem/leaf callus tissues in order to find out the optimal concentration of

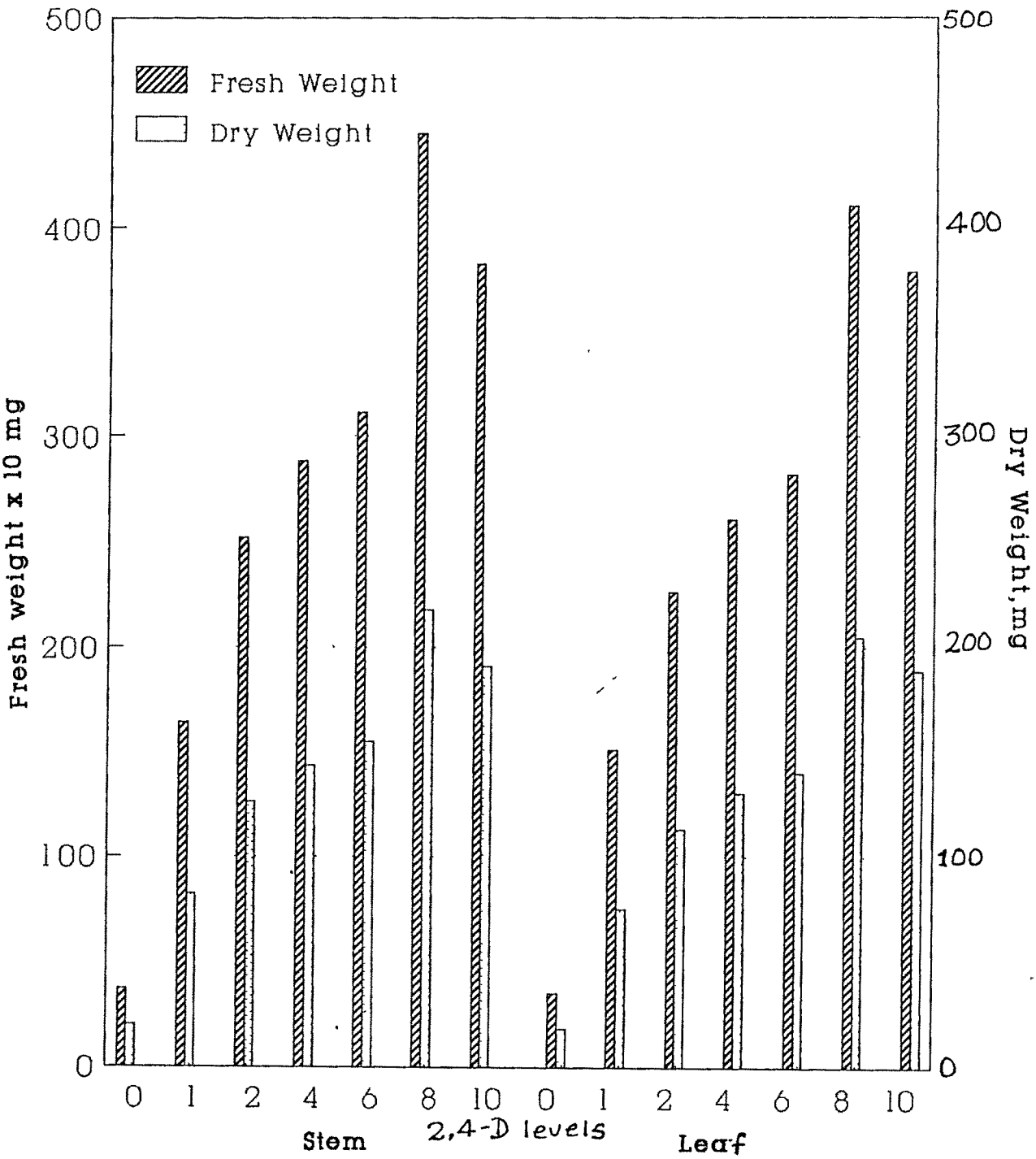
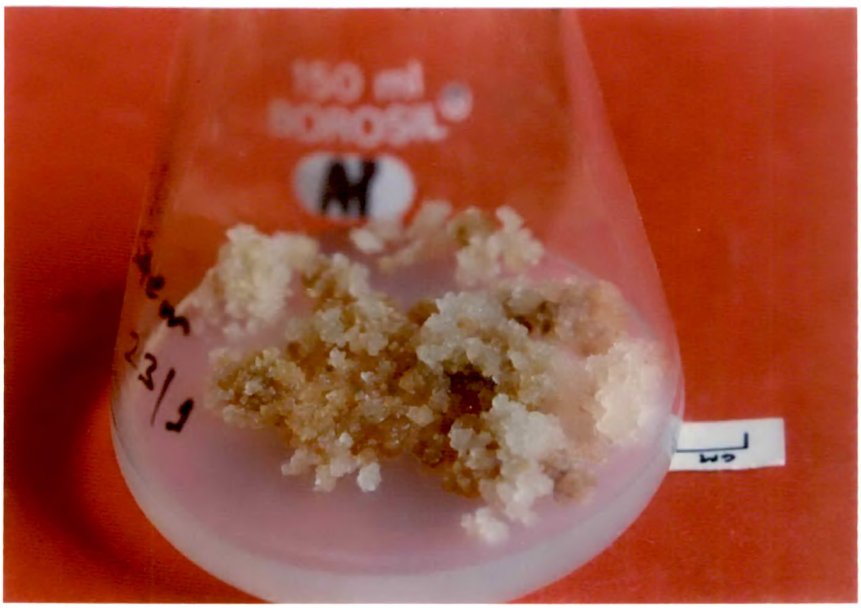


Fig. 15b Growth of callus tissues of stem and leaf at various concentrations of 2,4-D

Fig.16 Callus tissues of (a) stem and (b) leaf

Medium : MS + Sucrose (2%) + Kn (1 μM/l) + 2,4-D (8 μM/l)

Incubation : Four weeks at 25 $\pm$ 2°C in light



a

b

Fig. 16

sucrose required for their maximum biomass production.

Results after four weeks of incubation period revealed that sucrose being sole energy source was needed for callus growth as in its absence the callus did not survive. With sucrose incorporation in culture media at 1% and 2% level, there was a linear increase in the values of biomass production. However, highest values of fresh and dry weight of callus tissues were recorded at 3% sucrose which proved to be the optimal level (4962 ± 55 and 4820 ± 53 mg and 287 ± 20 and 279 ± 28 mg as fresh and dry weights of stem and leaf callus tissues respectively). At 4% sucrose level, there was a decline in fresh and dry weights in both the callus tissues (Fig. 17).

MS medium containing sucrose Kn (1 μM/l) and 2,4-D (8 μM/l) was therefore designed as "Standard Medium" for *B.diffusa* L. stem/leaf callus tissues.

Experiment 15 : Alkaloid profile of stem/leaf callus tissues

Callus tissues (300 ± 20 mg) induced from stem/leaf cultured on MS (30 ml) standard medium showed that growth of callus followed the normal pattern (Fig. 18). Callus tissues of both stem and leaf initiated growth during second and third week of culture period there after there was a decline in growth. However, the alkaloid profile of stem callus gave the indication of the presence of alkaloid with Draggendorf's reagent, but its quantity was very less and hence it could not be quantified. Leaf callus accumulated 0.007% of alkaloid, which on further incubation for four more weeks increased to 0.02%.

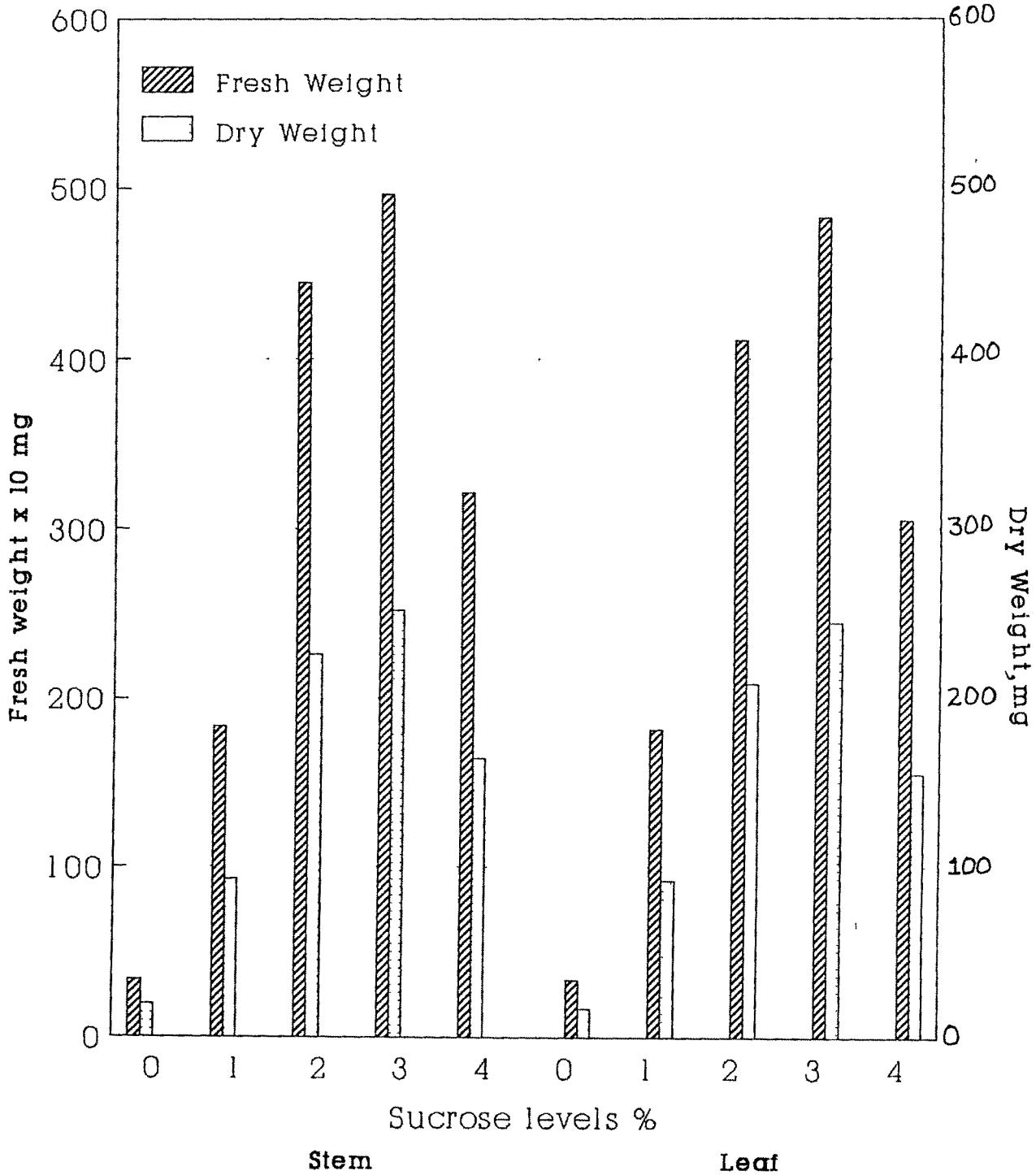


Fig. 17 Growth of callus tissues of stem and leaf at various concentratinos of sucrose

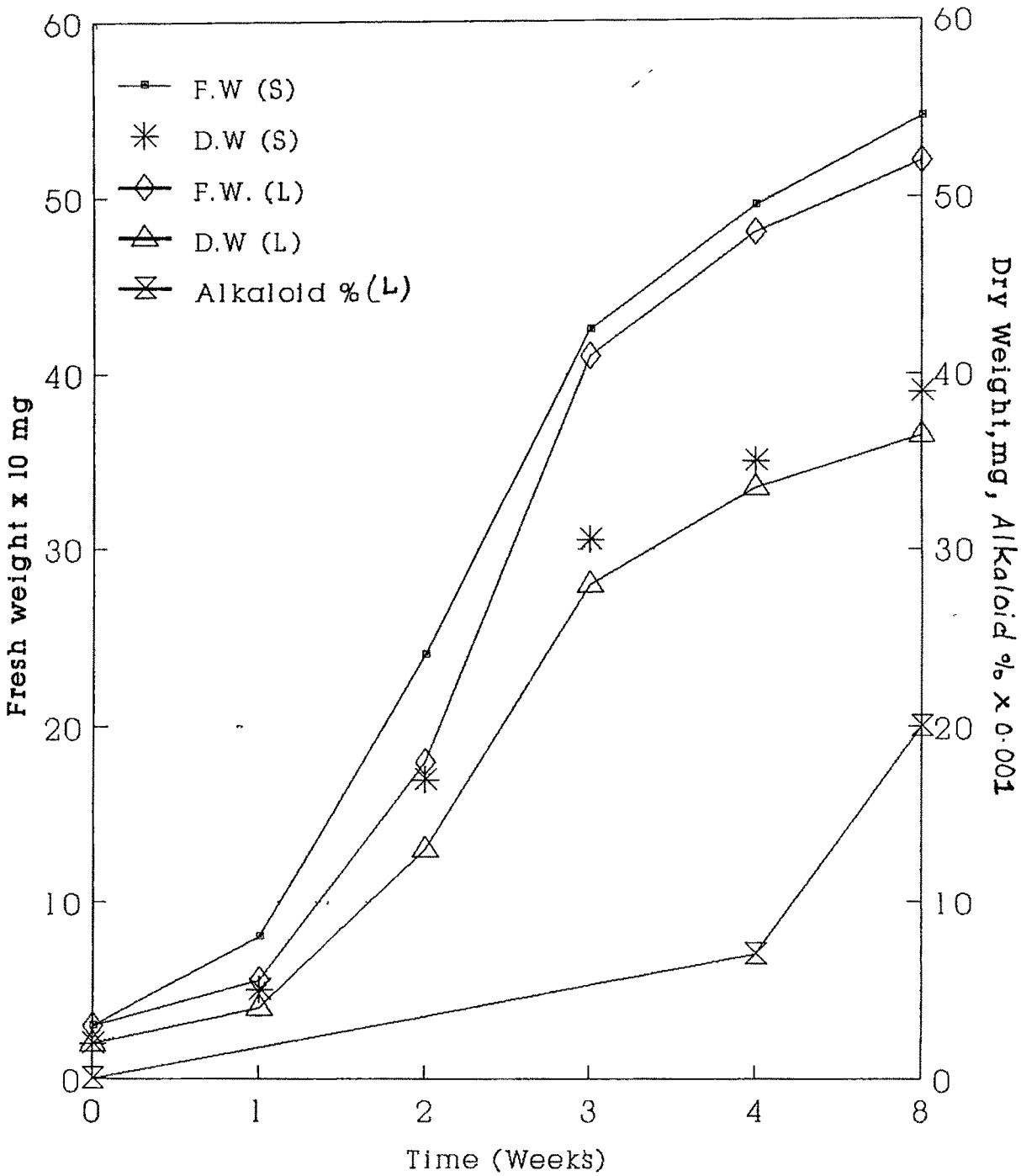


Fig.18 Growth pattern of callus tissues of stem (S) and leaf (L) with the alkaloid content

F.W. : Fresh Weight, D.W. : Dry Weight

Thus, it was evident from this experiment that stem/leaf callus tissues possessed biosynthetic ability for punarnavine alkaloid synthesis/accumulation.

Experiment 16 : Morphogenetic potential of callus cultures

Stem and leaf initiated callus tissues grown on standard medium were transferred to basal medium for a week to remove their carry over effects of cytokinin - auxin treatments. The morphogenetic potential of these callus tissues were examined by subjecting them to 3% sucrose with Kn (1 μM/l) in absence of 2, 4-D or in its presence at 0.01 or 0.05 or 0.1 or 0.5 μM/l levels. Callus tissues 1-2 cm in diameter, were transferred to above mentioned media and the flasks were kept in culture room at 25 $\pm$ 2°C in 16 h light (1000 lux).

Results recorded in Table XI shows that in absence of 2,4-D in the culture medium, stem as well as leaf initiated callus tissues differentiated into roots. The number of roots regenerated from stem callus were many and few of them were quite long (Fig.19 a). The leaf callus continued its growth for a short period and then differentiated into white roots with plenty of hairs (Fig. 19 b) at the end of four weeks experimental period.

With 2,4-D incorporation in the culture medium at 0.01 and 0.05 μM/l levels, there was no visible organ differentiation. However, stem callus turned into compact mass at the end of two weeks period. Histological observations of this compact callus showed that in the homogeneous mass of parenchymatous tissue, few cells have lost their nuclei and lignin was deposited on their walls. Lignin

Table XI : Morphogenic potential of callus tissues of *Boerhaavia diffusa* L.

Medium : MS + sucrose (3%) + Kn (1μM/l) + 2,4-D (0 - 0.5 μM/l)

Incubation : 4 weeks of culture period at 25 $\pm$ 2°C

Sr. No.	Levels of 2,4-D μM/l	Response	
		Stem Callus	Leaf Callus
1	00	R	R
2	0.01	NC	-
3	0.05	NC	-
4	0.1	-	-
5	0.5	-	-

Mean of six replicates

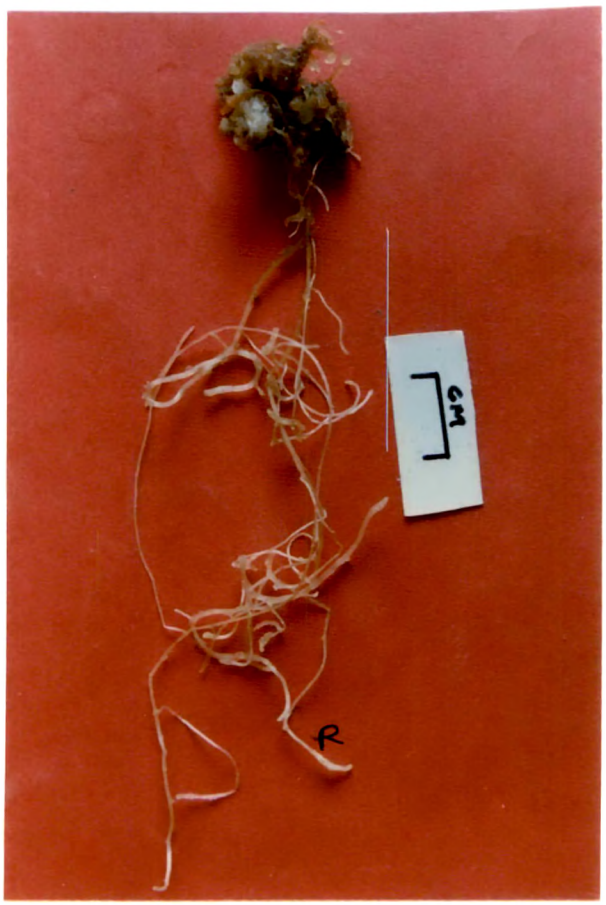
- No response

NC - nodular callus

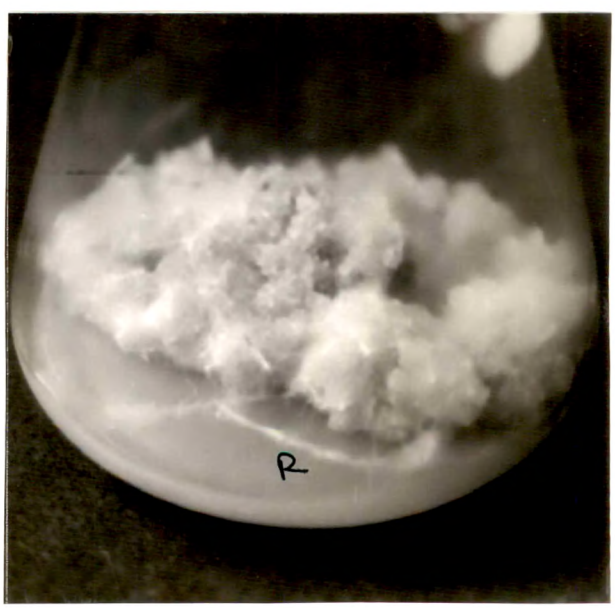
R - root

Fig.19 (a) Stem Callus differentiated into roots (R), (b) leaf callus differentiated into roots (R) after four weeks

Medium : MS + Sucrose (3%) + Kn (1 μM/l)



a



b

Fig. 19

deposition occurred in the form of spiral or reticulate manner (Fig.20 a,b). Infact, the differentiation of tracheary elements in the callus occurred when 2,4-D level of the culture medium was 0.01 μM/l. However, in callus cultured on 0.05 μM/l 2,4-D medium, more number of cells turned into tracheary elements was revealed (Fig. 20 c, d). This process of xylogenesis was in response to the auxin (2,4-D) incorporated in culture medium. Also lignin deposition occurred in the form of ridges on the original primary cell-wall which were of cellulose or hemicellulose material.

Stem callus, on further incubation for one more week turned distinctly nodular (Fig.21). Histological observations of each nodule indicated that it consisted of thin walled cells, each with prominent nucleus which were compactly arranged (Fig. 22). These nodules represented the growth centres having the capacity to develop into either roots or shoots as per the cytokinin auxin ratio of the culture medium. The stem callus subjected to higher 2,4-D levels *viz.*, 0.1 or 0.5 μM/l failed to differentiate and continued its growth. Hence, higher levels of 2,4-D were not given to other callus tissues. The leaf callus subjected to the above treatment failed to differentiate into tracheary elements as well as into visible organs.

For regeneration of plantlet from the nodular stem callus it was subjected to BAP and $AdSO_4$ containing media at various levels as seen in Table XII. Nodular portions of the callus tissues cultured on BAP containing media (0.5 μM/l to 4 μM/l levels) turned green due to the synthesis of chlorophyll, however, there was no shoot bud induction. With the incorporation of $AdSO_4$ in combination with (2

Table XII : Shoot-bud induction from stem callus cultures of

***Boerhaavia diffusa* L.**

Medium : MS + sucrose (3%) + BAP (0.5-4.0 μM/l) + $AdSO_4$ (0.5-4 μM/l)

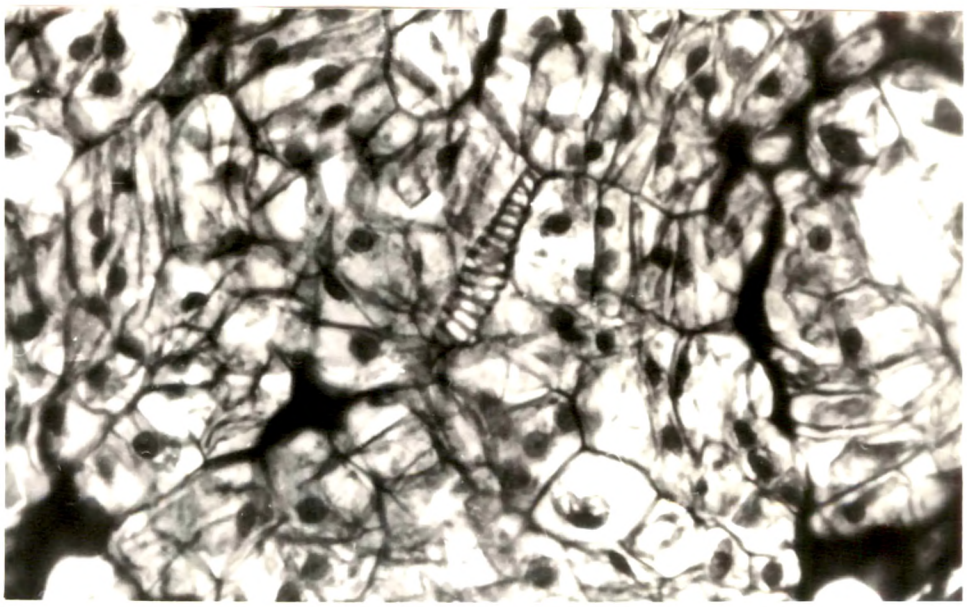
Incubation: Four weeks in culture room at 25 $\pm$ 2°C

Sr. No.	Levels of BAP	Levels of $AdSO_4$	Morphogenic response of callus Stem $\rightarrow$	Shoot Buds No.
1	0.5	-	-	-
2	1.0	-	Slightly green	-
3	2.0	-	Green	-
4	4.0	-	Green	-
5	2	0.5	Shoot bud	1-2
6	2	1.0	Shoot buds	5-6
7	2	2.0	Shoot buds	10-12
8	2	4.0	Shoot buds	4-5

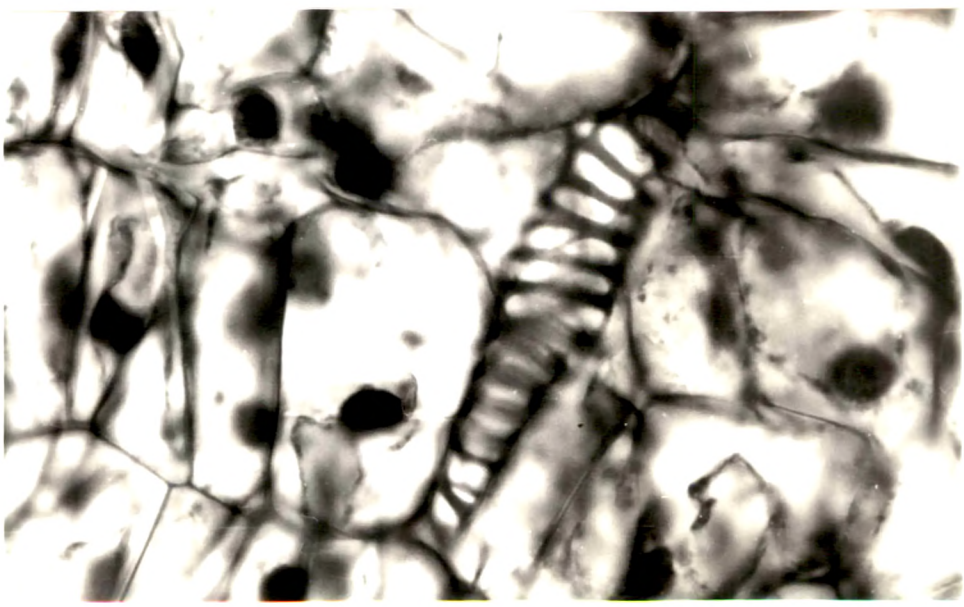
Mean of six replicates

- Nil

Fig.20 Lignin deposition in cells of stem callus (a) spiral (b) Raticulate (250 X)



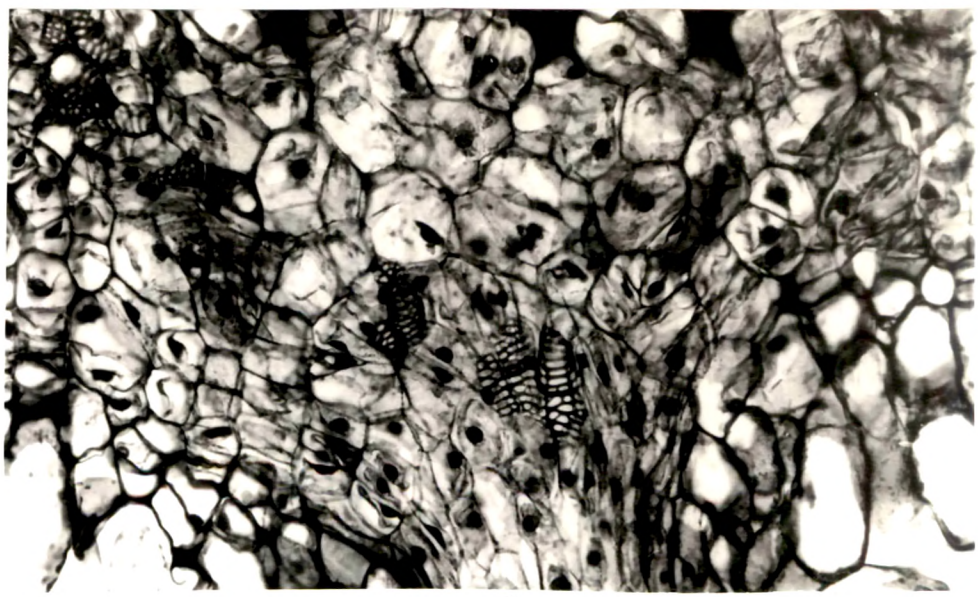
a



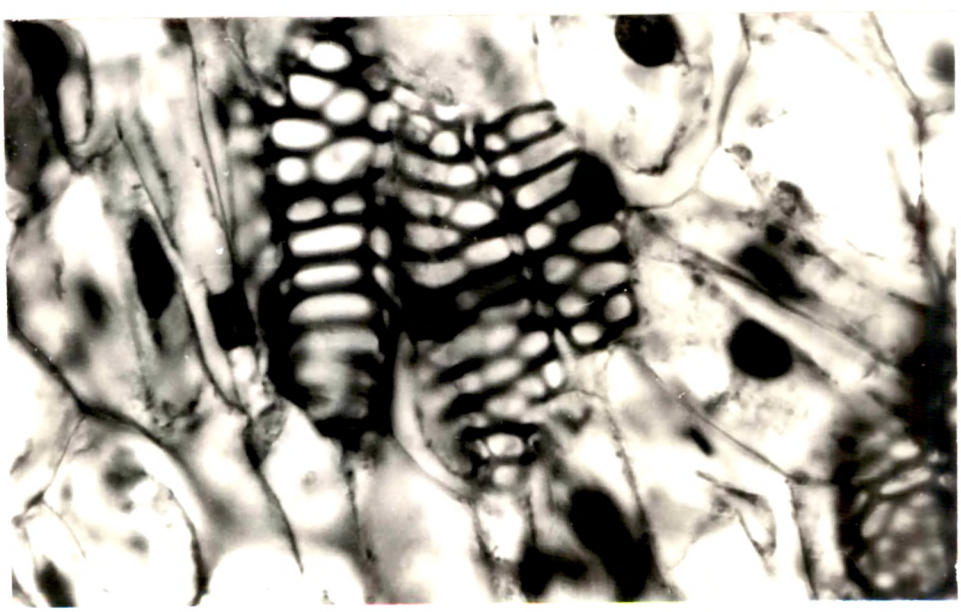
b

Fig. 20

Fig.20 Tracheary element in stem callus (c and d) 160 X



c



d

Fig. 20

Fig.21 Nodular stem callus

Fig.22 Photomicrograph of callus nodule with embryogenic cells (250 X)

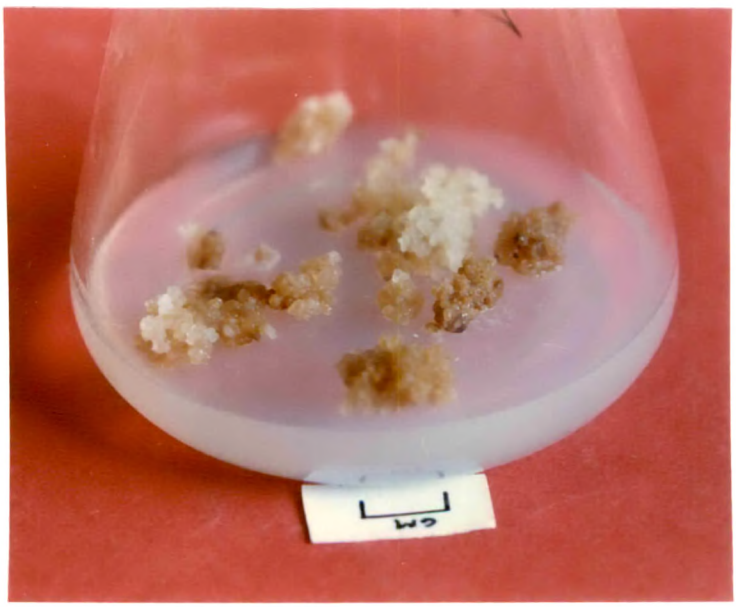


Fig. 21

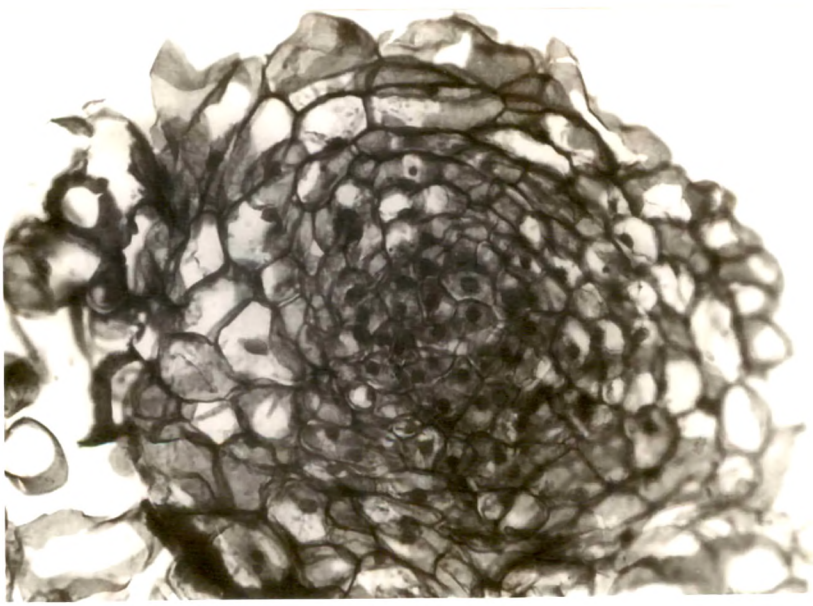


Fig. 22

μM/l) BAP, from the nodules of the stem callus, shoot buds were developed. Highest (10-12) shoot buds were produced when $AdSO_4$ level was 2 μM/l (Fig. 23). However, further increase in $AdSO_4$ level to 4 μM/l failed to enhance the number of shoot buds. These shoot buds grew well (Fig. 24 a). Each of the shoot bud was isolated and cultured on BM supplemented with NAA (0.01 μM/l), where it produced root. Thus regeneration of *B. diffusa* L. plantlet was achieved (Fig. 24 b).

It showed that the stem callus alone regenerated into plantlets while the leaf callus lacked this potential.

SECTION D : REGENERATIVE POTENTIAL OF EXCISED LEAVES

In this section, the regenerative potential of excised leaf segment of *B. diffusa* L. has been reported. The regenerated roots were grown in continuous cultures and their biosynthetic potential for punarnavine synthesis/accumulation was examined.

Experiment 17 : Regenerative ability of excised leaf segments

Leaves of garden grown *B. diffusa* L. `elite' plant were collected, first to fifth leaf from the apex in their serial order of development. They were washed under tap water and surface sterilized according to the procedures described in Chapter II, Materials and Methods. Each leaf was cut into segments and cultured on MS medium (30 ml) with sucrose (2%), basal medium-BM and BM supplemented with IAA at various levels (0.5 to 4 μM/l). Other experimental procedure remained the same.

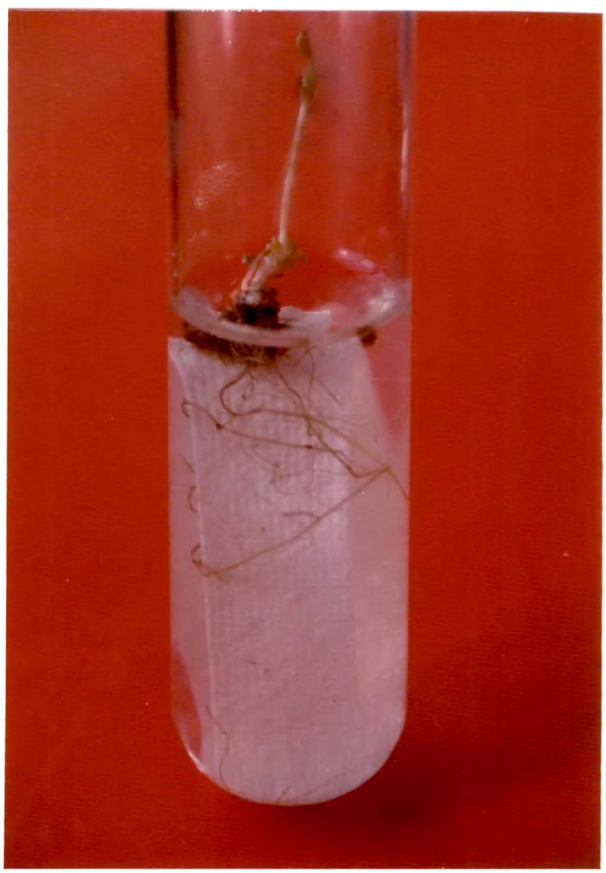
Fig.23 Shoot buds developed from nodular stem callus

Incubation : Four weeks on MS medium at 25 ± 2°C

Fig. 23

Fig.24 Plantlet regeneration from stem callus (a) and (b)

a



b

Fig. 24

Results (Table XIII) indicated that the third and fourth leaf segments cultured on BM turned black within one week of culture period. With the incorporation of IAA at 0.5 μM/l, roots regenerated from these leaf segments within one week culture period (Fig. 25). Highest number of roots, (17 roots) were regenerated from the leaf segments within four weeks of experimental period (Fig.26 a). The roots were 10 cm in length. Further increase in IAA level to 1 or 2 μM/l of culture medium, not only reduced the number of roots regeneration from 17 to 9 (1 μM/l) and 7 (2 μM/l) but also reduced their length to 5 and 3.5 cm respectively (Fig.26 b,c). Callus along with few roots regenerated when IAA level was 4 μM/l (Fig.26 d). Thus, it was apparent that the quantity of IAA present in the culture medium had pronounced effect on the morphogenetic behaviour of leaf segments. Histological studies during root regeneration from leaf segments showed that the root originated from phloem parenchyma of the vascular bundle (Fig. 27).

Experiment 18 : Punarnavine profile of regenerated roots

Roots having 0.5 μM/l IAA treatment were excised and cultured in liquid MS medium (40 ml) containing sucrose (2%), IAA 0.5 μM/l. Within four weeks, root clones were produced in all the replicates. They were examined for their alkaloid contents following the procedures described by Huber (1967) as given in Chapter II. The biomass production was 385 $\pm$ 7 mg and 23 $\pm$ 0.8 mg in terms of fresh and dry weights respectively (Table XIV). Their punarnavine content was 0.15% on dry weight basis. These roots were subcultured and at the end of eight weeks period the alkaloid content increased to 0.2% thereby indicating that regenerated roots also possess ability for alkaloid synthesis.

Table XIII : Regeneration of roots from the third leaf segments of *Boerhaavia diffusa* L.

Medium : MS + sucrose (2%) + IAA (0.5-4.0 μM/l)

Inoculum : Leaf segments of third leaf (2-3 pieces)

Incubation : 4 weeks of culture period at 25 ± 2°C

Sr. No.	Levels of Auxin μM/l	No.of regenerated roots	Length of roots (cm)	% response
1	00	-	-	-
IAA				
2	0.5	17	10 ± 1	80
3	1.0	09	5 ± 1.5	80
4	2.0	07	3.5 ± 0.5	90
5	4.0	04	1.5 ± 0.2	80
NAA				
6	0.5	-	-	-
7	1.0	-	-	-
8	2.0	-	-	-
9	4.0	-	-	-
2,4-D				
10	0.5	Callus + roots	-	-
11	1.0	Callus + roots	-	-
12	2.0	-	-	-
13	4.0	-	-	-

Mean of six replicates with standard deviation

- No response

Table XIV : Punarnavine profile of regenerated roots after four and eight weeks of culture period

Medium : MS (liquid) + sucrose (2%) + IAA (0.5 μM/l)

Inoculum : 10 roots with tips (1-2 cm) excised from leaf segments

Incubation : 8 weeks of culture period on gyratory shaker at 25 $\pm$ 2°C in culture room

Sr No.	Level of IAA μm/L	Fresh weight mg	Dry weight mg	Punarnavine %
After 4 weeks				
1	0.5	385 $\pm$ 7.6	23.0 $\pm$ 0.8	0.15
After 8 weeks				
2	0.5	569 $\pm$ 12	34.2 $\pm$ 2	0.2

Mean of six replicates with standard deviation

% on dry weight basis

Fig.25 Regenerated roots (R) from cultured leaf segments

Medium : MS + Sucrose (2%) + IAA (0.5 μM/l)

Incubation : One week culture period in light at 25 $\pm$ 2°C

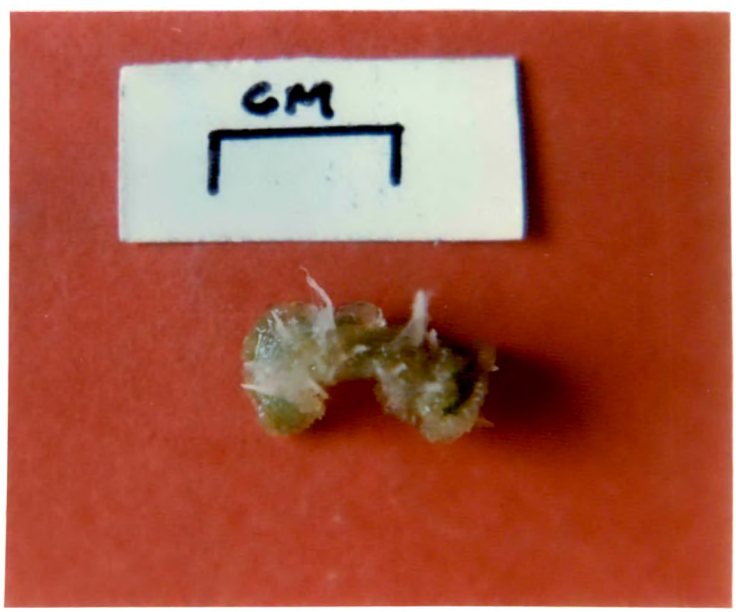


Fig. 25

Fig.26 (a) Profuse root regenerated from excised leaf segments of *Boerhaavia diffusa* on BM + 0.5 μM/l IAA

(b) Few roots regenerated on BM + 1.0 μM/l of IAA

(c) Roots regenerated on BM + 2 μM/l of IAA

(d) Callus with short roots on BM + 4 μM/l of IAA

Incubation : Four weeks at 25 $\pm$ 2°C in light

a

b

c

d

Fig. 26

Fig.27 T.S.of cultured leaf segment showing root initiation (R) from Phloem parenchyma (Ph) 160 X, Ep - Upper epidermis, P - palisade, VB - Vascular bundle

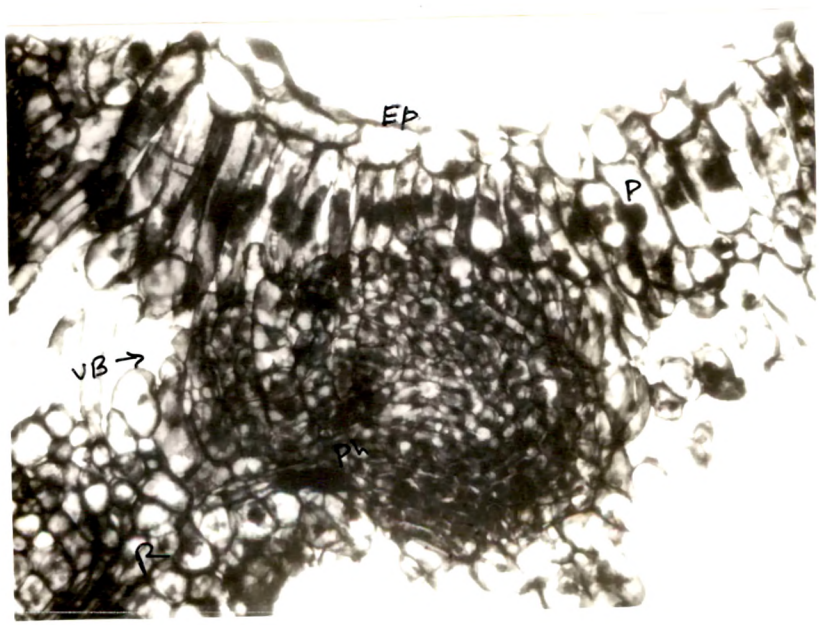


Fig. 27

PART II

Achyranthes aspera L.

Achyranthes aspera L. a common annual indigenous herb is known for its medicinal importance. Its medicinal value lies in the alkaloids, achyranthine and two others, synthesized during the metabolic activities of the plant. It is used in a number of Ayurvedic formulations and in the anti-fertility drug preparations. Besides,the ash of this plant is used in the Kshaarsootra preparation.

The present study includes *in vivo* screening of *A. aspera* L. plants for the presence of alkaloid/s for the selection of `elite' superior plants. Screening of individual organs of the elite plant of *A. aspera* L. was conducted to ascertain the site of synthesis/accumulation of the alkaloid achyranthine.

SECTION A: *IN VIVO* SCREENING OF PLANT

Experiment 19 : Extraction, isolation and identification of achyranthine alkaloid from *Achyranthes aspera* L.

Healthy mature *A. aspera* plants from the M.S. University Campus (Fig.28) were collected, washed under tap water, rinsed in distilled water and dried in oven at 40°C. Each plant was powdered and alkaloid containing compounds were extracted in ethanol from the powder sample (20 g) in soxhlet following the procedures described by Kapoor and Singh (1967) as given in Chapter II, Materials and Methods.

The extract was used for preparatory TLC and the spots developed were tested with Dragendorff's reagent for the presence of alkaloid. All the spots turned orange indicating that they were of the alkaloid. The spots were eluted with the solvent and

once again 2 ml of aliquots in duplicate were spotted on TLC plates along with the authentic sample of achyranthine. The Rf values of the alkaloid from the extract and of the authentic sample were the same (0.72) which confirmed that the isolated alkaloid, achyranthine from the plant was in its pure form . (Fig.29).

Experiment 20 : Alkaloid profile of *Achyranthes aspera* L. plant and its organs

Fertile plants of *A.aspera* were collected washed under tap water and dried in oven at 40°C. A single dried plant was powdered. Another plant was separated into its individual organs *viz.*, stem, leaves, flowers and all these organs were powdered separately. Achyranthine was extracted from 0.2 g of each of the powdered samples employing the procedure described by Huber (1967) as given in the Chapter II, Materials and Methods.

Results displayed in Table XV showed that the whole plant contained maximum quantity of achyranthine (0.11%) calculated on dry weight basis. Amongst the individual organs of a plant, stem accumulated the highest quantity of achyranthine (0.05%) followed by leaves (0.03%), flowers (0.02%) and roots (0.01%). From these results it was evident that no single organ of this plant was specialized for the alkaloid accumulation and hence the plant as a whole should be employed for extraction of achyranthine which could be used in drug preparation.

Table XV : Achyranthine contents in a plant and its individual organs

Sr.No.	Plant/Plant organs	Achyranthine %
1.	Whole plant	0.11
2.	Roots	0.01
3.	Stem	0.05
4.	Leaves	0.03
5.	Flowers	0.02

% on dry weight basis

Fig.28 *Achyranthes aspera* L. plant

Fig. 28

Fig.29 TLC of achyranthine (a) authentic (b) isolated

Fig. 29

Experiment 21 : Quantitative estimations of Achyranthine from plants of different localities

This experiment was conducted to find out the alkaloid profile of the plants growing in different localities. Fertile plants of the M.S.University Campus, Sama, Nandesari and G.I.D.C Makarpura areas from their natural habitat were uprooted, washed and dried at 40°C. They were powdered and alkaloid was extracted from each of the powder sample employing Huber's (1967) procedure. The results presented in Table XVI, show that the highest quantity of achyranthine (0.12%) was present in plants of Sama area, followed by the M.S.University Campus plant (0.11%). Amongst the industrial areas, Nandesari plants accumulated the lowest quantity (0.04%) of alkaloid. The results indicated that alkaloid contents of same age plants varied with the variation in localities. This suggested that to obtain sufficient amounts of alkaloid for drug preparation, a uniform population of these plants should be grown from a selected superior `elite' genotype under uniform environmental conditions.

Based on the findings given above *A. aspera* L. plants grown in Sama locality were considered as an `elite' superior plants and they were used for further experimental work.

SECTION B : CALLUS CULTURES AND PLANTLET REGENERATION

The present section deals with callus cultures of various organs of *A. aspera* plant. Callus tissues of various organs of `elite' plant were established by culturing

Table XVI : Alkaloid contents in *Achyranthes aspera* L. plants growing in different localities

Sr.No.	Locality	Achyranthine %
1.	The M.S.University Campus	0.11
2.	Sama	0.12
3.	Nandesari	0.04
4.	GIDC Makarpura	0.08

% on dry weight basis

them individually on MS medium and supplemented with appropriate doses of cytokinin auxin. The nutritional/hormonal levels were standardized supporting highest biomass values of callus tissue in terms of fresh and dry weights. Further, the capacity of callus tissues for synthesis/accumulation of achyranthine was evaluated. Capacity of plantlet regeneration from these callus cultures was also examined in order to develop uniform plantation of *A. aspera* which could supply higher quantities of achyranthine.

Experiment 22 : Callus induction from stem, leaf and spike of `elite'

Achyranthes aspera L.

In order to induce callus tissue, stem/leaf/spike pieces from elite *A. aspera*, after sterilization according to the procedure described in Chapter II, were inoculated on MS medium (30 ml) containing sucrose (2%) and supplemented with Kn (0-4 μM/l) in combination with various levels (0-6 μM/l) of IAA/NAA/2,4-D. Culture flasks were incubated at 25 $\pm$ 2°C in culture room in light 16 h (1000 lux).

Table XVII indicates that in absence of Kn and IAA/NAA/ 2,4-D no callus was induced from any of the cultured explants thereby indicating the need of phytohormones for callus induction.

In presence of Kn at 1 and 2 μM/l level with IAA 2 μM/l, roots were induced from stem (Fig. 30) and also from leaf explants.

Treatments of Kn in combination with NAA at all the levels, failed to induce callus from stem/leaf/spike.

Table XVII : Effect of Kn in combination with auxin (IAA/NAA/2,4-D) on callus induction from stem/leaf/floral bud explant of *Achyranthes aspera* L.

Sr. No.	Auxin μM/l	Kn	Stem				Leaf				Spike			
			0.5	1.0	2.0	4.0	0.5	1.0	2.0	4.0	0.5	1.0	2.0	4.0
	IAA													
1	00		-	-	-	-	-	-	-	-	-	-	-	-
2	0.5		-	-	-	-	-	-	-	-	-	-	-	-
3	1.0		-	-	-	-	-	R	R	-	-	-	-	-
4	2.0		-	R	R	-	-	R	R	-	-	-	-	-
5	4.0		-	-	-	-	-	R	R	-	-	-	-	-
6	6.0		-	-	-	-	-	-	-	-	-	-	-	-
	NAA													
7	0-6				-				-				-	
	2,4-D													
8	00		-	-	-	-	-	-	-	-	-	-	-	-
9	0.5		+	-	-	-	-	-	-	-	-	-	-	-
10	1.0		++	++	+	-	+	+	++	-	++	++	++	-
11	2.0		++	+++	+++	-	++	+	+++	-	+	++	+++	-
12	4.0		+	++	++++	-	+	+	++++	-	+	++	+++	-
13	6.0		-	-	-	-	-	-	+	-	-	-	+	-

Mean of six replicates
- No response, + denotes amount of callus induced, R roots

However, in response to Kn in combination with 2,4-D it was noticed that Kn at 0.5 or 1 μM/l with 2,4-D at 0.5, 1 or 2 μM/l level induced callus from the cut ends of the explant, it was yellowish in colour and covered half to three fourth of the explant at the end of four weeks. But in presence of 2 μM/l Kn in combination with 2,4-D at 4 μM/l callus was induced at much faster rate. The callus completely covered the stem and leaf explants (Fig 31 a, b). In spike, callus was induced from axis portion (Fig.31 c), but none of the floral parts were able to induce callus. Further increase in 2,4-D level to 6 μM/l, the callus induction capacity was declined in the explant. Hence Kn at 2 μM/l level in combination with 4 μM/l of 2,4-D was selected as the suitable combination of phytohormones for callus induction.

The stem/leaf callus tissues were used for further experimental work.

Experiment 23 : Determination of optimal levels of Kn and 2,4-D for the highest biomass production of callus tissues

(i) Optimal Kn level :

Stem and leaf callus cultures (300 $\pm$ 20 mg) were inoculated on MS medium (30 ml) containing sucrose (2%), 2,4-D (2 μM/l), and supplemented with Kn at 0 to 4 μM/l levels.

Results noted after four weeks of incubation showed that in absence of Kn there was a slight increase in fresh and dry weights of callus tissues. Addition of Kn to the culture medium (0.5 μM/l) induced callus growth which was maximum when Kn level was 2 μM/l. The fresh weights of stem and leaf callus tissues recorded were

Fig.30 Roots regenerated from stem pieces of *Achyranthes aspera* L.

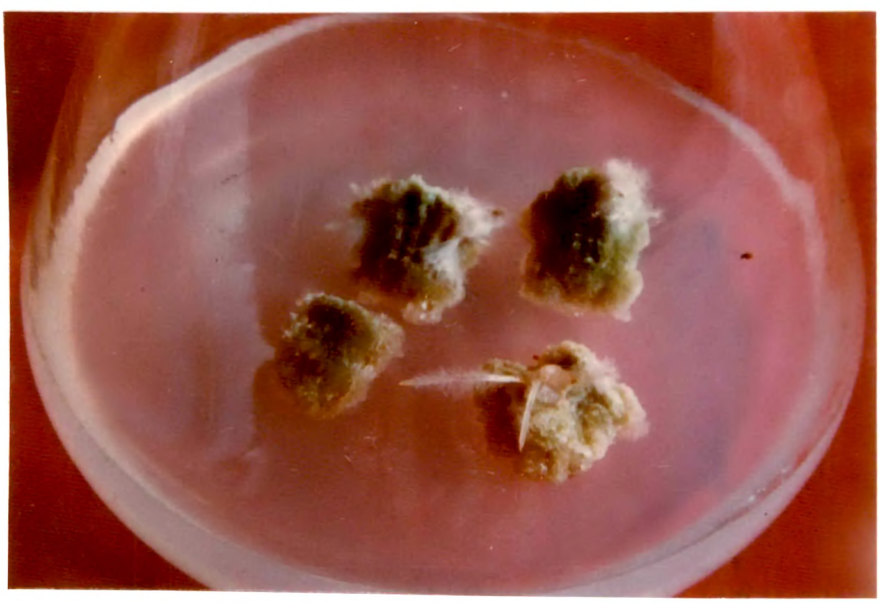


Fig. 30

Fig.31 Callus induction from (a) stem (b) leaf (c) spike axis

a

b

c

Fig. 31

897 ± 20 mg, 932 ± 22 mg and dry weights 66.3 ± 7 mg and 68.9 ± 6 mg respectively (Fig 32 a).

Callus tissues were yellow coloured but turned to brown colour with the culture period. Further increase in Kn level caused decline in these growth parameters thereby proving that Kn at 2 μM/l level was the optimal for callus growth.

(ii) Optimal 2,4-D level

Out of three auxins tested for callus induction, 2,4-D was found suitable since in almost all the replicates the callus tissues were initiated. Hence, in this experiment optimal level of 2,4-D required for stem/leaf callus tissues for the highest biomass production was determined. In absence of 2,4-D there was slight increase in fresh and dry weights of these cultures (Fig 32 b). However, with 2-4-D incorporation both the callus tissues (stem/leaf) improved their growth in linear manner upto 4 μM/l level. Their fresh weights recorded were, stem callus 1050 ± 35 mg, leaf callus 1287 ± 40 mg and dry weights 73 ± 12 and 90 ± 18 mg respectively (Fig.32 b). Further increase in 2,4-D levels to 6 μM/l reduced these growth parameters. Thus,2,4-D at 4 μM/l was considered as the optimal level required for the highest biomass production. Hence, it was proved that optimal level of Kn and 2,4-D were 2 μM/l and 4 μM/l respectively for the highest biomass production of stem and leaf callus tissues (Fig.33 a, b).

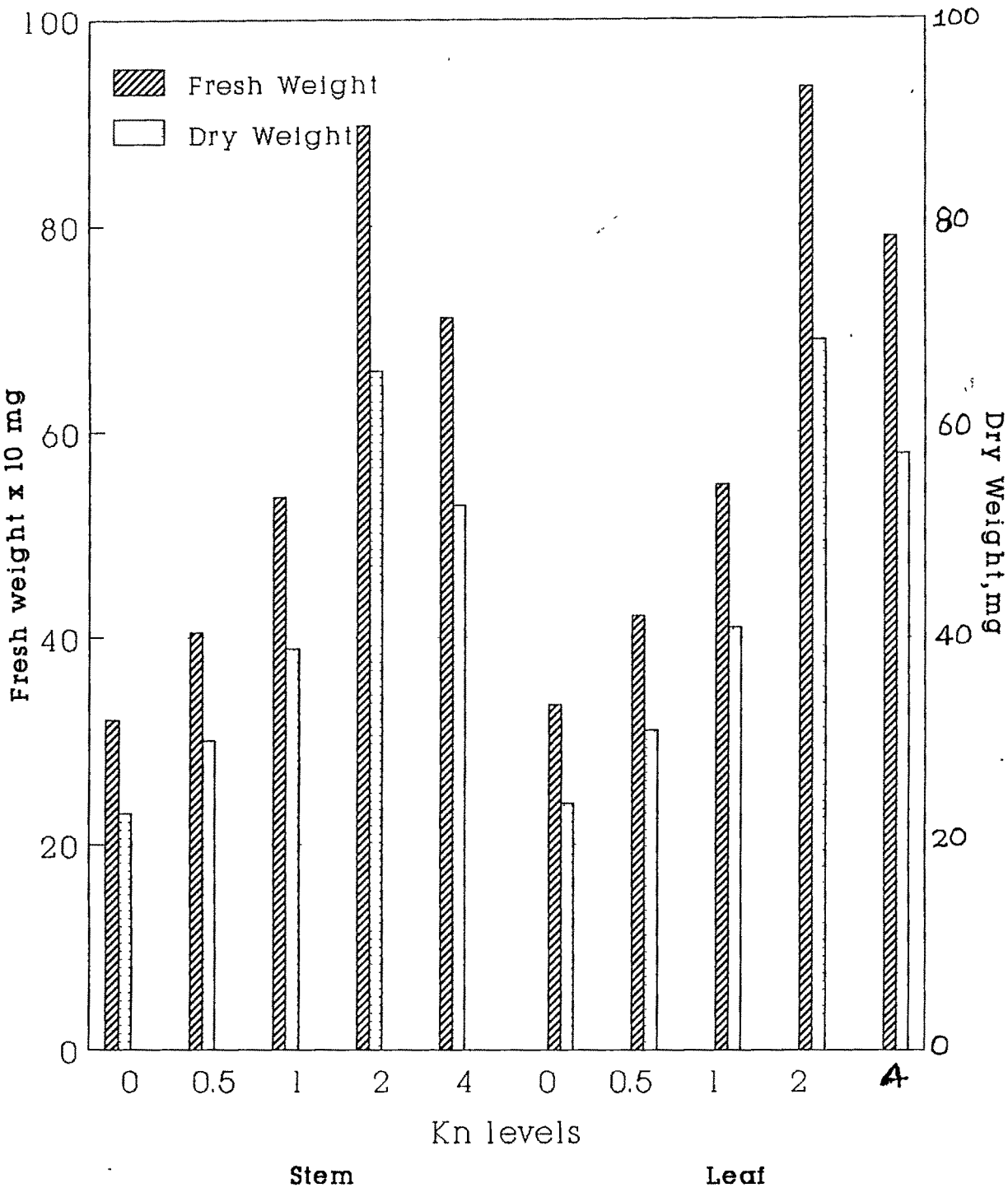


Fig.32a Growth of callus tissues of stem and leaf at various concentrations of Kn

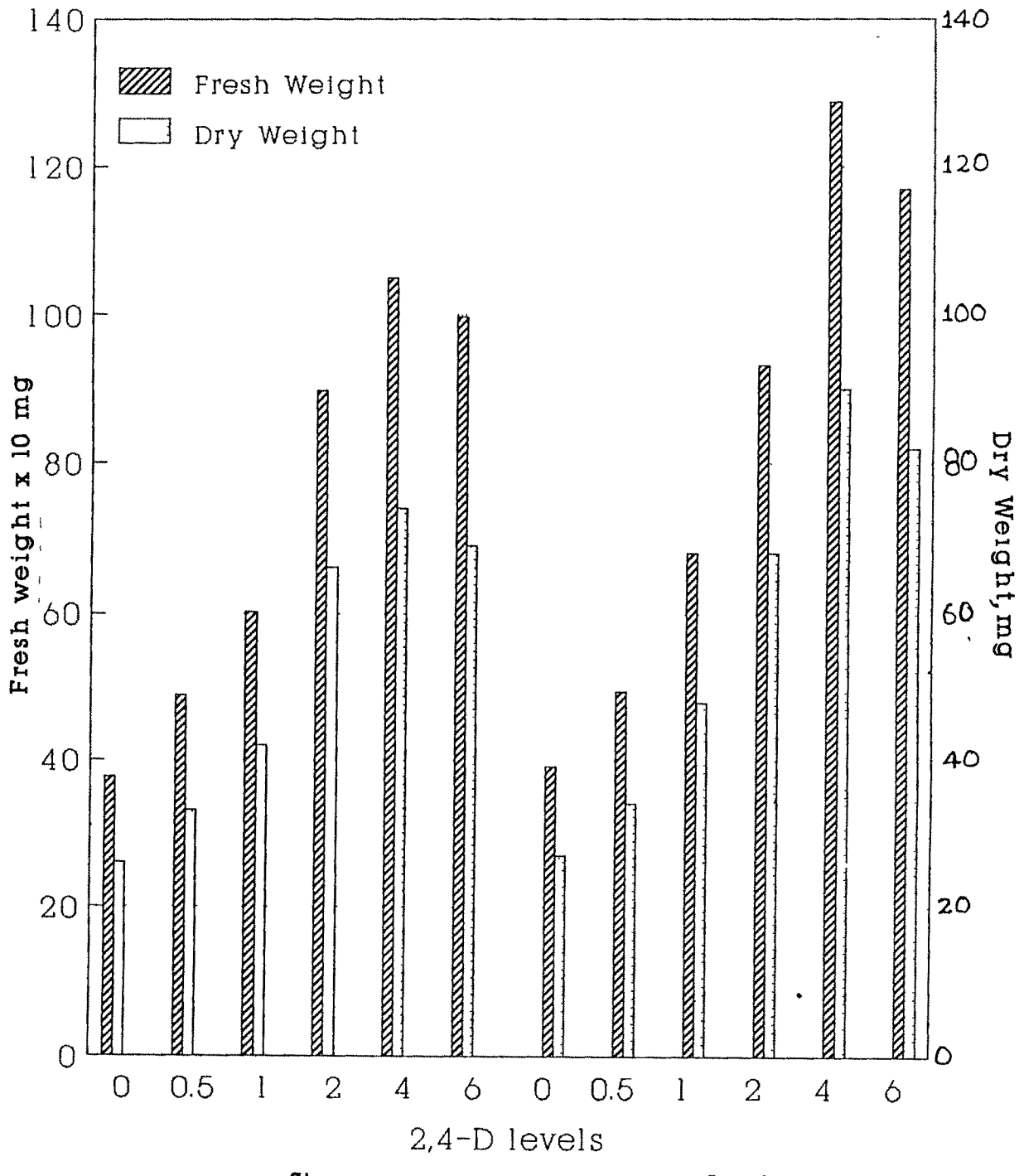


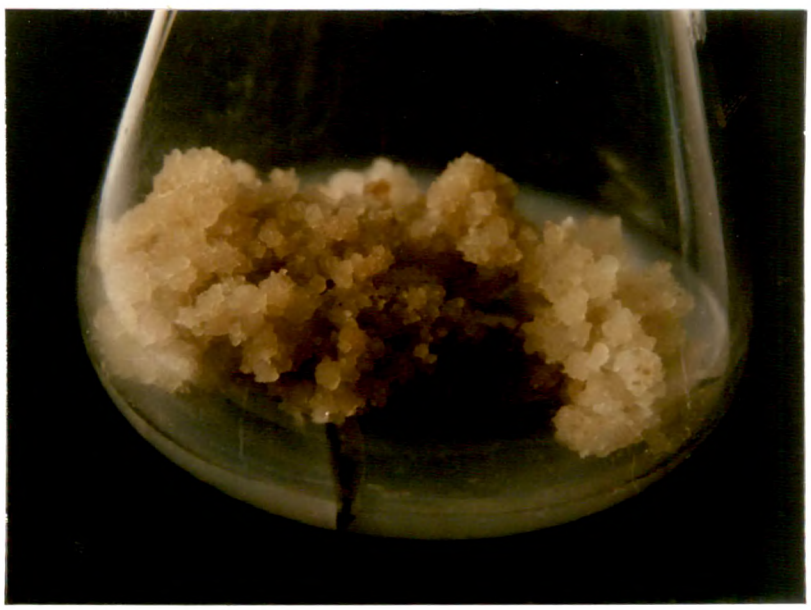
Fig.32b Growth of callus tissues of stem and leaf at various concentrations of 2,4-D

Fig.33 Profuse growth of callus tissues of (a) stem (b) leaf

Medium : MS + Sucorse (2%) + Kn (2 μM/l) + 2,4-D 4μM/l

Incubation : Four weeks at 25 $\pm$2°C in light

a



b

Fig. 33

Experiment 24 : Determination of optimal sucrose level for biomass production of stem/leaf callus

The carbohydrate incorporated in the culture medium forms the main energy source, its required concentration needs to be estimated. In the present study, sucrose was used as the carbohydrate hence, its optimal level for the highest production of biomass of callus in terms of fresh and dry weights were estimated.

Stem/leaf callus tissues (300 ± 20 mg) maintained on BM for a week were incubated on MS media (30 ml) supplemented with Kn (2 μM/l), 2,4-D (4 μM/l) and sucrose at various levels 0, 1, 2, 3 and 4%. Culture flasks were incubated as per the standard procedures. Results recorded (Fig. 34) after four week period indicated that in absence of sucrose callus ceased to grow and it turned brown and ultimately died.

Addition of sucrose at 1% level improved callus growth in terms of fresh and dry weights. Highest growth was recorded at 3% sucrose level (1432 ± 35 mg, 1692 ± 42 mg fresh weights and 97 ± 12, 115 ± 15 mg dry weights of stem and leaf callus respectively). Further increase in sucrose level to 4%, declined the growth parameters, thereby 3% sucrose proved to be the optimal level required for the highest biomass production of stem callus (Fig.35) and also the same level was found optimal for leaf callus. MS medium containing sucrose (3%), Kn (2 μM/l) and 2,4-D (4 μM/l) was therefore designed as "Standard Medium" for *A. aspera* L. stem/leaf callus tissues.

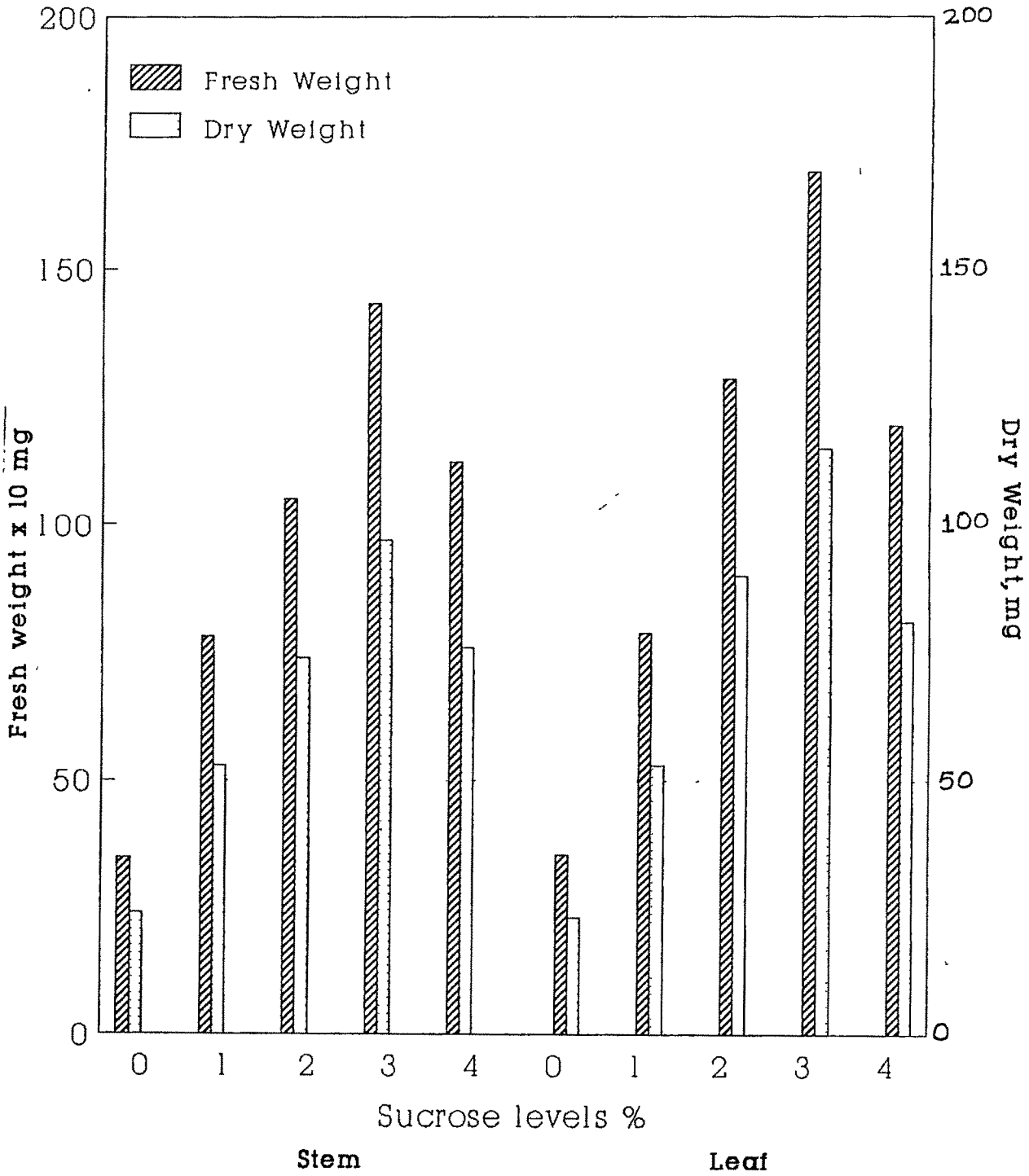


Fig.34 Growth of callus tissues of stem and leaf on various concentrations of sucrose

Fig.35 Profuse growth of stem callus

Medium : MS + Sucrose (3%) + Kn (2 μM/l) + 2,4-D (4 μM/l)

Incubation : Four weeks at 25 $\pm$ 2°C in light

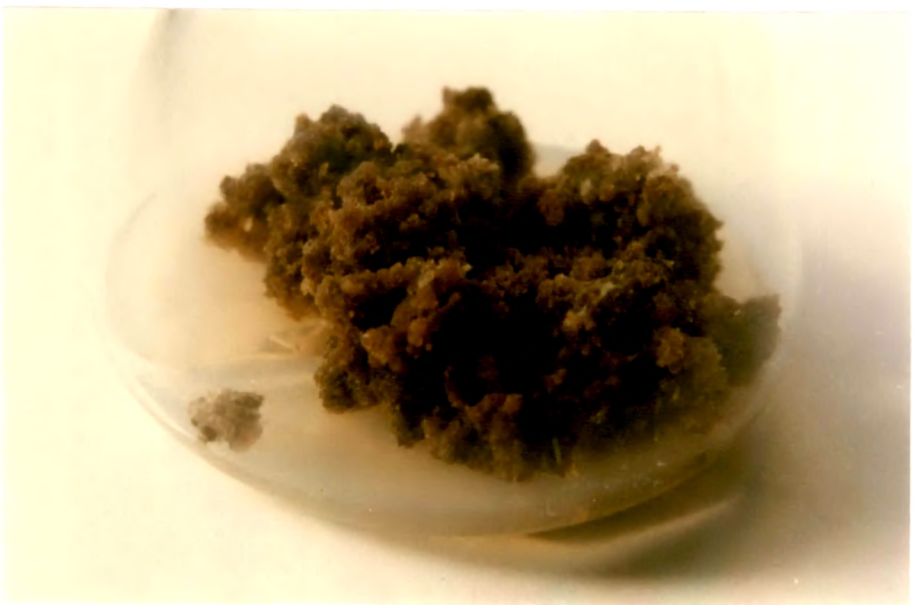


Fig. 35

Experiment 25 : Growth kinetics and alkaloid profile of stem/leaf callus cultures

About 300 ± 20 mg stem/leaf callus tissues were transferred to standard MS medium (30 ml) and other experimental conditions were kept constant.

(i) Growth kinetics

Results recorded at weekly interval indicated that during first week,callus tissues showed slight growth which improved during the second and third week period. During the fourth week, however, the growth once again declined (Fig.36). The graph of growth values in terms of fresh and dry weights plotted against time (weeks) developed sigmoid curve which proved that the growth was as expected.

(ii) Alkaloid profile of callus cultures

Callus of stem/leaf grown on standard medium when screened for achyranthine at the end of four weeks period indicated just it's presence. Hence, these callus cultures were grown on medium containing low levels of 2,4-D as well as other auxins viz., IAA, NAA. Stem/leaf callus tissues (300 ± 20 mg) were transferred to MS medium containing sucrose (3%), Kn (2 μM/l) and 2,4-D/IAA/NAA (1 μM/l). Culture flasks were maintained as per standard procedure.

Results of the alkaloid analysis conducted according to the procedures described by Huber (1967) given in Chapter II, indicated that at the end of four weeks period, stem callus developed on 2, 4-D supplemented medium contained negligible amount of achyranthine alkaloid while in callus grown on IAA and NAA supplemented media achyranthine quantities were 0.007 and 0.002%.

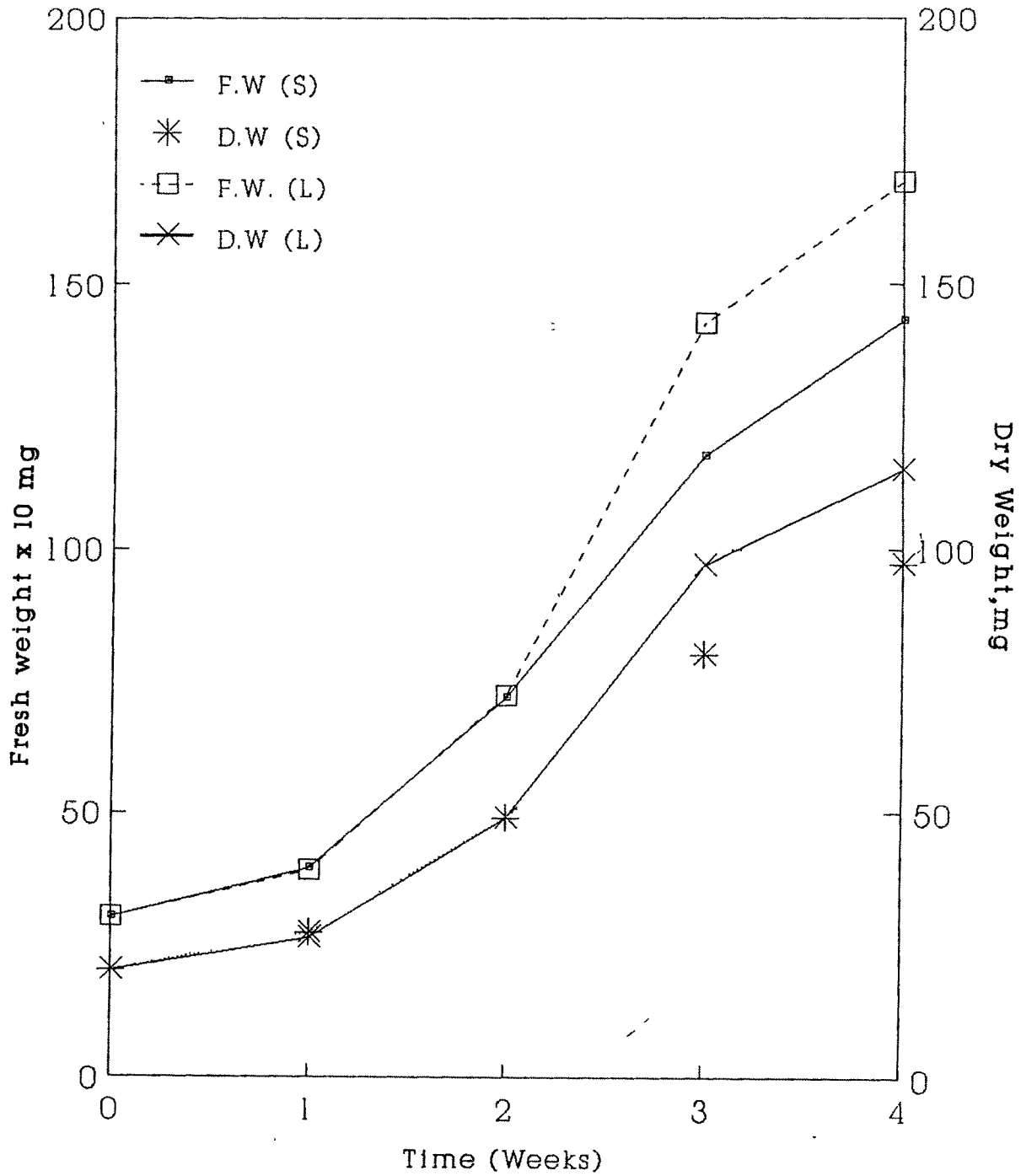


Fig.36 Growth pattern of callus tissues of stem(S) and leaf (L) on standard medium

F.W. : Fresh Weight, D.W. : Dry Weight

In order to find out whether the increase in culture period could support synthesis/accumulation of achyranthine, the stem callus cultures were subcultured on fresh media (30 ml) of the same composition. At the end of four more weeks - that was total period of eight weeks - showed that the biomass of stem callus had increased slightly in terms of fresh and dry weights but there was an appreciable increase in achyranthine quantities (Table XVIII).

Results of leaf callus grown on 2, 4-D containing medium also showed that at the end of four weeks period, only the presence of achyranthine was detected. However, in IAA and NAA supplemented media the quantities of achyranthine were 0.003 and 0.001% respectively. To enhance the culture period to eight weeks, the tissues were transferred onto fresh media of the same composition. The alkaloid achyranthine was 0.001% in 2,4-D supplemented medium while it is 0.005 and 0.002% in IAA and NAA supplemented medium respectively.

This experiment indicated that the stem/leaf callus tissue possessed the ability for the synthesis of achyranthine and it varied according to the auxin treatment.

Experiment 26 : Morphogenetic potential of callus cultures

This experiment was conducted to regenerate a plantlet from stem/leaf induced callus cultures. Stem/leaf callus tissues (1-2 cm in diameter) were transferred to MS medium containing varied levels of 2,4-D (00/0.01/0.05/0.1/0.5 μM/l) with 3% sucrose and Kn (2 μM/l). Culture flasks were incubated as described earlier.

Table XVIII : Effect of Auxins on alkaloid profile of callus cultures of *Achyranthes aspera* L.

Medium : MS + sucrose (3%) + Kn (2 μM/l) + IAA/NAA/2,4-D (1 μM/l)
Incubation : 8 weeks of culture period at 25 ± 2°C in culture room
Inoculum : Callus tissues 300 ± 20 mg Fresh Weight, 20 ± 2 mg Dry Weight

Sr. No.	Name of Auxin	Stem			Leaf		
		Fresh Wt. (mg)	Dry. Wt. (mg)	Achy ranth ine %	Fresh Wt. (mg)	Dry Wt. (mg)	Achy rant ine %
After 4 weeks							
1.	IAA	610±15	42±5	0.007	652±12	43.6±5	0.003
2.	NAA	465±9	32±2	0.002	510±10	34.2±2.5	0.001
3.	2,4-D	1432±38	97.3±15	PD	1682±43	115±22	PD
After 8 weeks							
4.	IAA	679±20	50.93±6	0.01	732±15	55.13±6.5	0.005
5.	NAA	507±17	38.03±3.2	0.005	565±12	42.38±5	0.002
6.	2,4-D	1587±30	119.5±12	0.002	1810±45	135.7±17	0.001

Mean of six replicates with standard deviation

% on dry weight basis, PD Presence detected

Results recorded showed that in absence of 2,4-D or at its lower levels (0.01, 0.05 μM/l) the stem callus became compact with the formation of nodules (Fig.37 a), however, there was no change in the leaf callus tissue. But in no case any shoot/root was differentiated from the nodular stem callus. Higher levels of 2,4-D (0.1, 0.5 μM/l) failed to induce nodules in stem callus. Histological observations of nodular stem callus indicated that each nodule consisted of cells with prominent nuclei and they were arranged in compact masses (Fig. 37 b,c). These were the meristematic growth centres.

The capacity of these growth centres developed in stem callus for organogenesis was evaluated by culturing them on MS medium (30 ml) containing sucrose (3%) with Kn (2 μM/l) and BAP at various levels (1,2,4 μM/l). Cultural conditions were maintained uniform as per previous experiments.

It was observed that the nodular stem callus became greenish because of development of chlorophyll (Fig. 38) when BAP level was 4 μM/l. At lower levels of BAP (1 and 2 μM/l), no chlorophyll synthesis occurred in the nodular callus.

The greenish nodules were transferred to medium (30 ml) containing BAP (4 μM/l) in combination with $AdSO_4$ (1,2,4,6 μM/l) and these cultures were incubated at 25 $\pm$ 2°C in culture room at 16 h photoperiod (1000 lux).

The greenish nodules in presence of 2 μM/l of $AdSO_4$ developed into 2-3 shoot buds while increasing $AdSO_4$ level to 4 μM/l, the number of shoot buds increased to 6-7 in number (Table XIX). Further increase in level of $AdSO_4$ to 6 μM/l however, declined the formation of shoot-buds. These shoot bud were isolated and

Table XIX : Shoot bud induction from stem callus

Medium : MS + Sucrose (3%) + Kn (2 μM/l) + BAP (4-6 μM/l) + $AdSO_4$ (1-6 μM/l)

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

Sr. No.	Levels of BAP μM/l	Levels of $AdSO_4$ μM/l	Morphogenetic response shoot buds
1.	4	1	-
2.	"	2	2-3
3.	"	4	6-7
4.	"	6	1-2

Mean of six replicates

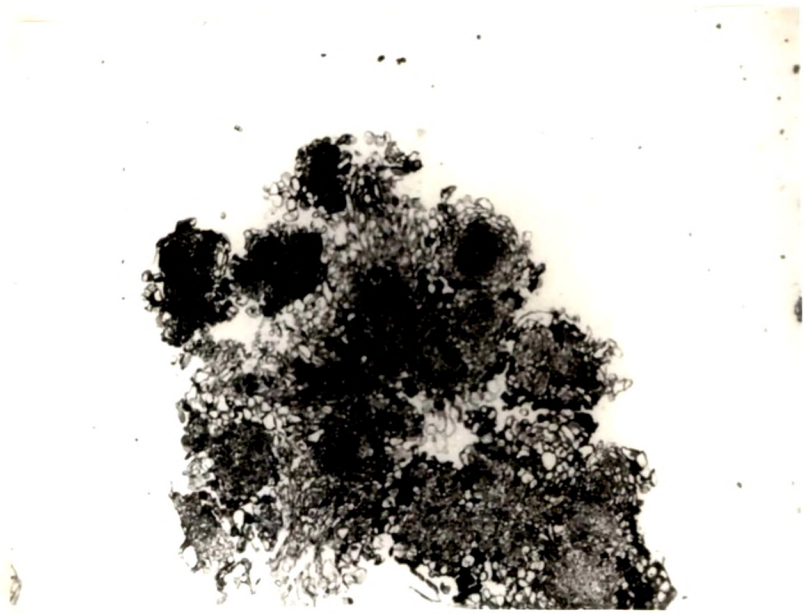
Fig.37 (a) Nodular Stem Callus

(b) Photomicrograph of nodular callus with growth centers (100 X)

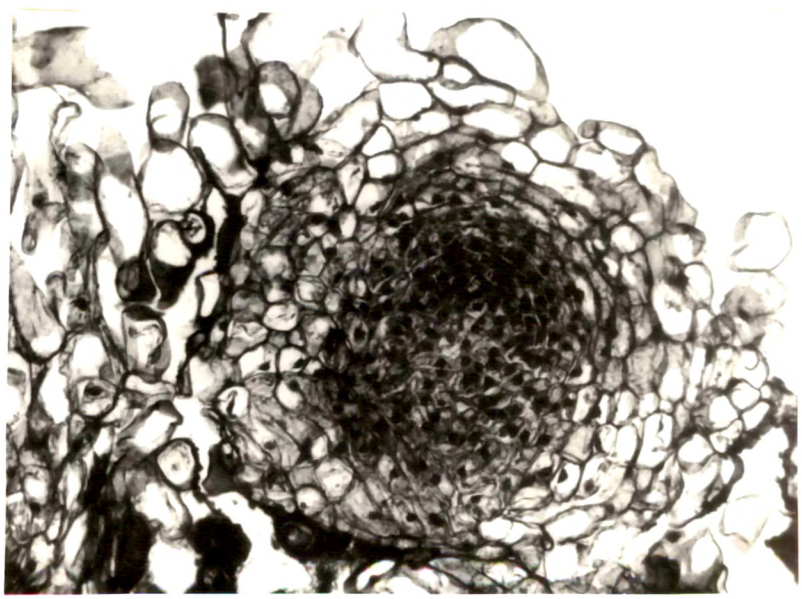
(c) Photomicrograph of callus nodule with embryogenic cells (250 X)

a

Fig. 37



b



c

Fig. 37

Fig.38 Green nodular stem callus

MS + Sucrose (3%) + Kn (2 μM/l) + BAP (4 μM/l)

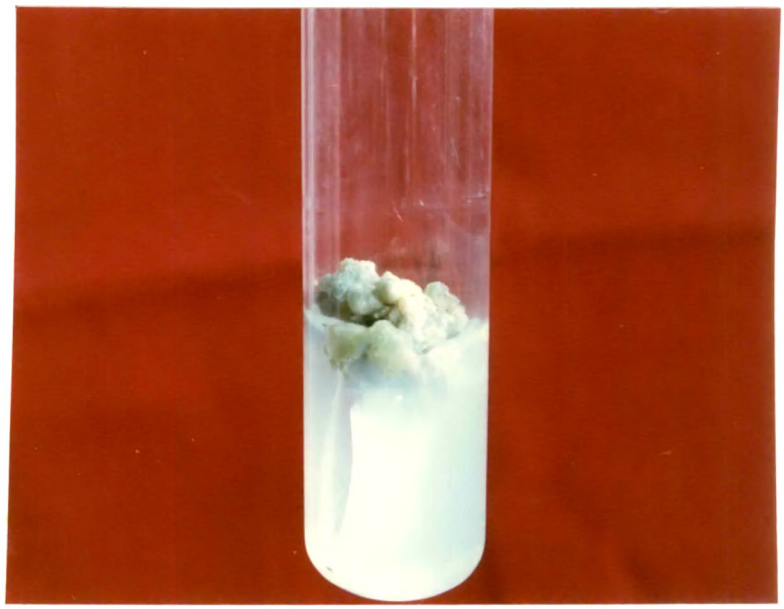


Fig. 38

transferred to NAA (0.01 μM/l) containing medium, where it grew well and developed roots, thus the formation of a plantlet was achieved (Fig. 39).

SECTION C : REGENERATIVE POTENTIAL OF EXCISED LEAVES

To evaluate the capacity of leaf segments of *A. aspera* L. for regeneration, the present experiment was conducted.

Experiment 27 : Regenerative potential of leaf segments of *Achyranthes aspera* L.

Leaves from `elite' *A. aspera* L. plants first to fifth from the apex were collected in flask containing water. They were washed under tap water, disinfected as per the procedures described in the chapter II Materials and Methods. They were transferred to sterile petri-dish and after cutting they were inoculated on MS medium (30 ml) containing sucrose (2%) and IAA/NAA/2,4-D at various levels (0.0 to 4 μM/l). Flasks were incubated as described earlier.

Results recorded indicated that leaf segments cultured on auxin free medium turned black without regeneration of any organs, within one week culture period (Table XX). In presence of IAA (0.5 μM/l), profuse roots were regenerated all over the leaf segment (Fig. 40 a). Each of the root reached to 7 $\pm$ 0.2 cm in length at the end of four weeks period. Increase of IAA to 1 or 2 μM/l proportionately reduced the number of roots regenerated to 3 and 2, besides their length also was reduced to 6 $\pm$ 0.5 cm and 4.0 $\pm$ 0.2 cm respectively (Fig. 40 b,c). At 4 μM/l of IAA, only short roots without lateral roots were regenerated from leaf segments (Fig. 40 d).

Table XX : Regeneration of roots from the third leaf segments of *Achyranthes aspera* L.

Medium : MS + Sucrose (2%) + IAA (0.5-4 μM/l)

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

Sr No.	Levels of IAA μM/l	No.of regenerated roots	Length of roots cm	No.of laterals per root	Response %
1.	00	-	-	-	-
2.	0.5	4	7 $\pm$ 0.2	14 $\pm$ 2	60
3.	1.0	3	6 $\pm$ 0.5	12 $\pm$ 1	80
4.	2.0	2	4 $\pm$ 0.2	6 $\pm$ 1	50
5.	4.0	4	2.5 $\pm$ 0.5	-	50

Mean of 6 replicates

- No response

Fig.39 Plantlet regenerated from stem callus

Fig. 39

Fig.40 Regeneration of roots from leaf segments of *Achyranthes aspera* L.

(a) Profuse root formation on BM + 0.5 μM/l IAA

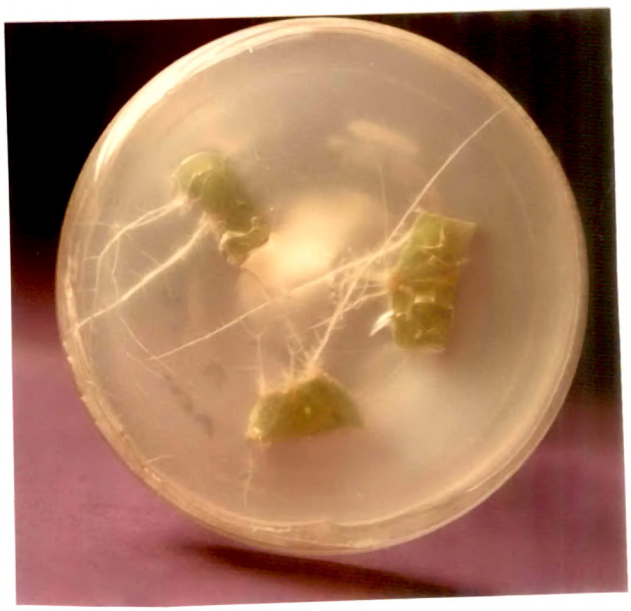
(b) Few roots regenerated on BM + 1 μM/l of IAA

(c) Roots regeneration on BM + 2 μM/l of IAA

(d) Roots regeneration on BM + 4 μM/l of IAA

Incubation : Four weeks in light at 25 $\pm$ 2°C

a



b

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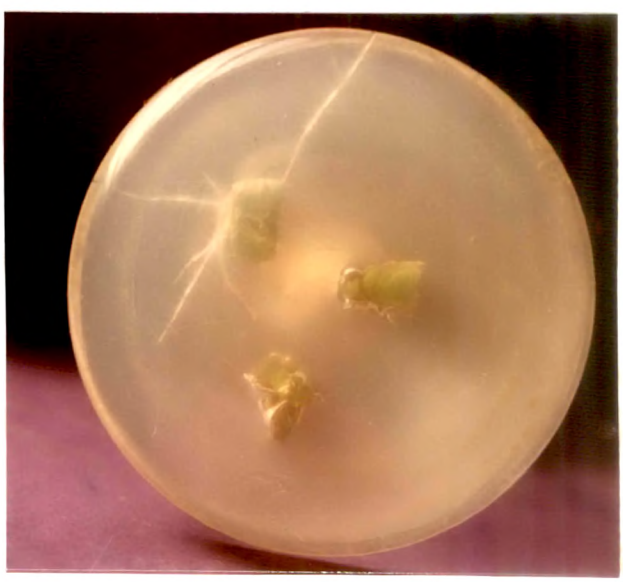

d

Fig. 40

Leaf segments in response to NAA treatment at various levels failed to regenerate roots/shoots. Leaf segments treated to 2,4-D at various levels developed callus only. In no case any regeneration occurred from them.

Thus, it was clear that IAA at low levels was competent to regenerate roots alone, in excised leaf segments.