CHAPTER - 3

RESULTS

DALBERGIA SISSOO ROXB.

RESULTS

Dalbergia sissoo Roxb. is a deciduous timber yielding and medicinally important tree. The medicinal importance of this plant lies in the chemical constituents synthesized as a result of the metabolic processes which occur during its growth and development. These chemicals include flavonoids / alkaloids / steroids which get accumulated in the various organs of the plant.

SECTION A : *IN VIVO* STUDIES

This section deals with the *in vivo* experimental work conducted on *D.sissoo* plant for the presence of flavonoids, in particular quercetin. Samples of stem-bark and leaves of about 50 year old tree of *D.sissoo* growing in the M.S. University Arboratum and from trees growing in the different localities of Baroda were collected and screened for the presence of quercetin. This medicinally important flavonoid was extracted, isolated, identified from the plant material and also quantified. An authentic sample of quercetin obtained from Sigma

Chemicals (U.S.A.) was used as reference sample. Amongst these plants, an 'elite' superior tree of *D.sissoo* based on the highest quercetin content was to be identified and used for *in vitro* experimental studies.

Experiment 1 : Selection of 'elite' *D.sissoo* plant

Healthy stem-bark and leaf samples were collected from about fifty year old plants of *D.sissoo* growing in M.S.University of Baroda, Arboratum (Fig.1 a&b), Subhanpura, Channi Jakat Naka and Refinery Township. These samples were washed under tap water, rinsed in distilled water, air dried at room temperature, powdered ,stored in glass bottles and were numbered.

Powder samples of stem-bark and also of leaves (40 gm) were extracted in a soxhlet (48 h) using 200 ml of methanol (80%)as solvent for the isolation of flavonoids. The procedures described by Harborne (1978) as given in detail in Chapter II, Materials and Methods were followed. The solvent was distilled off, finally the residue obtained was re-dissolved

Fig. 1 (a) A tree of *Dalbergia sissoo* Roxb. growng in M.S. University Arboratum

(b) Flowering twig

a

b

Fig. 1

in water and filtered. The filtrate was hydrolysed with 7% HCl on a water-bath until solid mass was produced which was dissolved in ethylacetate. The ethylacetate fraction was isolated using separating funnel and solidified by evaporating the solvent. This residue was re-dissolved in the same solvent and loaded in the form of a band on the chromatographic paper for the isolation of flavonoid/s using acetic acid (30%) as the solvent system. The authentic sample of quercetin was also loaded on the chromatogram. Under UV light,a yellow coloured band was observed in the extract of the sample (Fig.2). Similar yellow coloured band was also developed in the authentic sample of quercetin under UV light indicating the presence of quercetin in the plant samples under investigation.

The isolated fractions from stem-barks as well as leaves collected from all the four plants growing in different localities when screened as per the above mentioned procedures indicated that quercetin was present in all of them. Besides, the compounds isolated from these samples when subjected to UV spectrophotometric analysis, two

Fig.2 Paper chromatogram of quercetin (Y) isolated from stem-bark

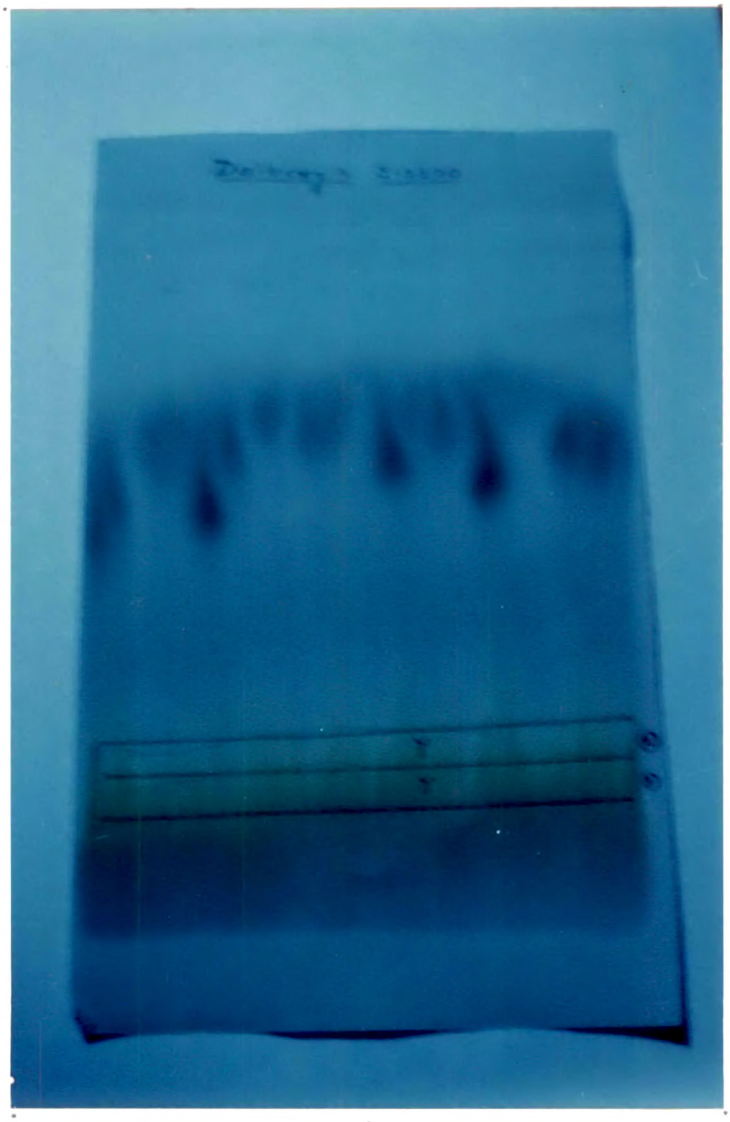


Fig. 2

superimposable spectra,one of authentic sample of quercetin (1) and second of quercetin isolated from leaf sample (2) were produced (Fig.3). Further, confirmation of quercetin was done by gas chromatographic analysis (Fig.4).

Quantification of the isolated quercetin from each sample was done according to the procedures described by Block and Durrum (1958) as given in Chapter II, Materials and Methods.

All the four stem-bark samples collected from *D.sissoo* plants growing in various localities showed the presence of quercetin. However, as their quantities of the quercetin being very low,they could not be quantified.

Results of Table II showed that leaf samples of *D.sissoo*, M.S.University Arboratum plant synthesized/ accumulated 1.25% of quercetin while the leaves of Subhanpura area growing plant accumulated 0.90% of quercetin when calculated on percentage dry weight basis. Leaf samples of

Fig.3 UV absorption spectra of quercetin
1) Authentic sample
2) Isolated from leaves of *D.sissoo*.

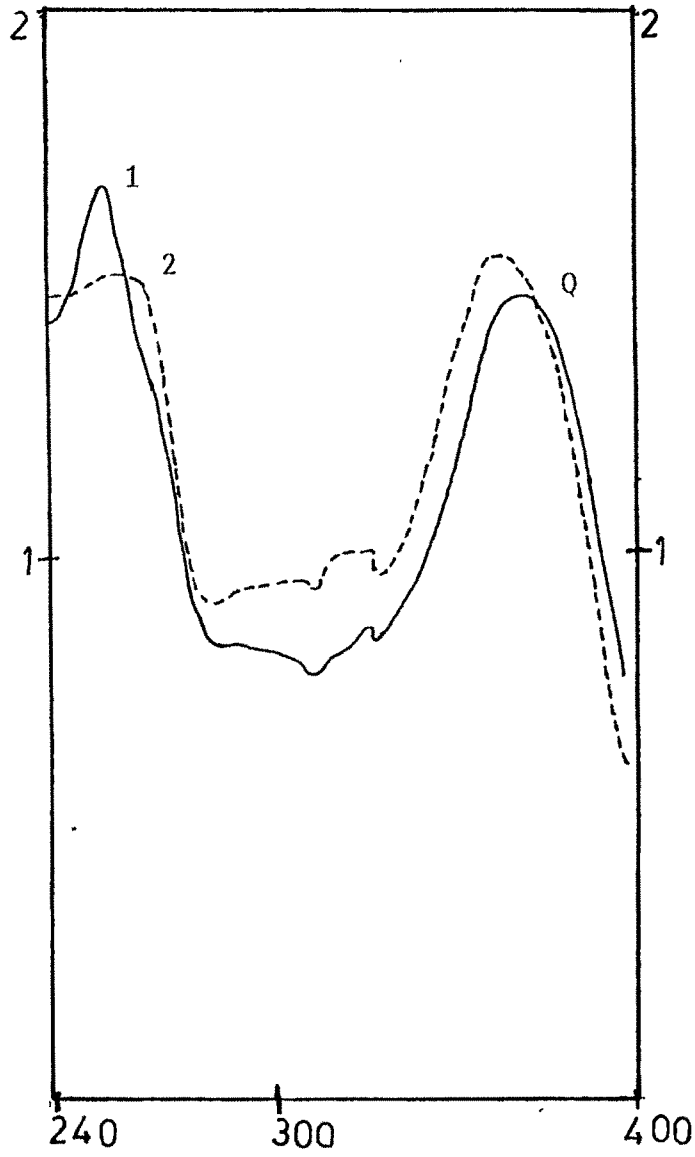


Fig. 3

Fig.4 Gas chromatograph of quercetin isolated from leaves

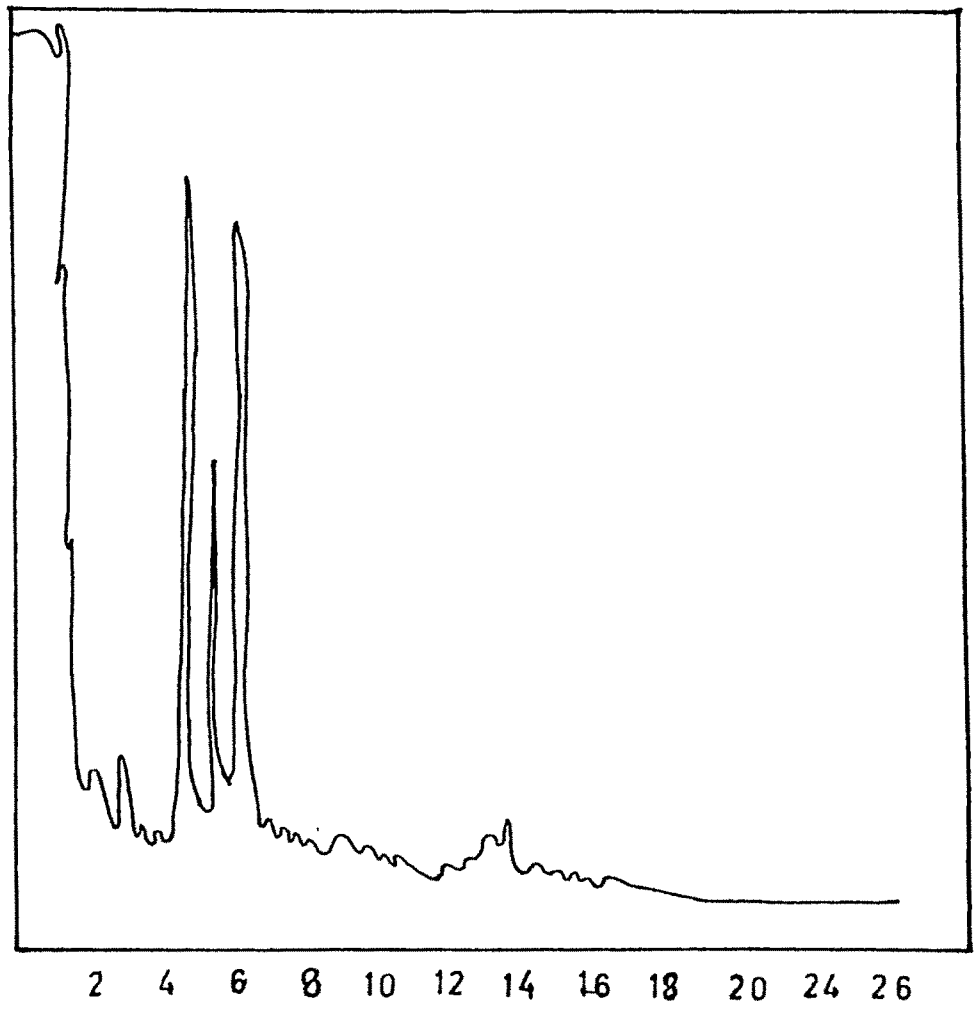


Fig. 4

TABLE II : Quercetin profile in leaf samples of *Dalbergia sissoo* Roxb.

Sr. No.	*D.sissoo* growing in different localities	Quercetin content (%)
1	M.S. University Arboratum	1.25
2	Subhanpura	0.90
3	Channi Jakat Naka	0.75
4	Refinery Township	0.65

% dry weight basis

Channi Jakat Naka area growing plant showed 0.75% of quercetin. Lowest quantity of quercetin, 0.65% was noted from leaves of *D.sissoo* plant growing in Refinery Township.

Results of this experiment showed that stem-bark and leaves of *D.sissoo* synthesized/ accumulated quercetin. Quantitative estimations revealed that very low quantities of quercetin were accumulated in all the four stem-bark samples and hence, they could not be quantified. Leaves of all the four plants synthesized/ accumulated appreciable amount of quercetin, highest quantity (1.25%)was found in the M.S. University Arboratum plant leaves. Based on this result, an Arboratum growing *D.sissoo* plant was selected as an 'elite' superior plant.

SECTION B : *IN VITRO* STUDIES

This section describes the experimental work conducted for standardization of the procedures for rapid multiplication and propagation of selected 'elite' *D.sissoo* plant employing tissue culture techniques. Axillary buds of the elite

plant were used as explants. In order to achieve rapid multiplication of this species through its axillary buds, hormonal, nutritional besides other cultural parameters have been standardized.

I. AXILLARY BUD SPROUTING

Experiment 2 : Effect of cytokinins (Kn/BAP) alone or in combination on axillary bud sprouting

In order to induce sprouting of axillary buds into numerous shoot-buds-the propagules-they were collected from the 'elite' *D.sissoo* plant and were subjected to Kn and BAP treatments alone or in combinations.

a) Effect of Kn

Healthy nodal segments with a axillary bud, second, third, fourth and fifth from the apex of a branch in serial order of its development were collected from an 'elite' *D.sissoo* plant. They were washed under tap water and subjected to surface sterilization procedures as described in Chapter II, Materials and Methods. Each bud was inoculated in Erlenmeyer flask (150 ml) containing MS basal medium(30ml) with sucrose (3%) supplemented with Kn (0,1,2,4,6 and 8µM/l), pH-5.7 gelled with agar (0.8%). Culture flasks were incubated at 25±2°C in culture room in 16 h photoperiod (1000 lux).

The axillary bud, grown on basal medium-medium without Kn- turned black within one week culture period. But, the bud grown on 1μM/l of Kn containing medium continued its growth and developed into single shoot (70%) at the end of four week culture period. Increasing the level of Kn to 2 μM/l, growth of the bud into a shoot was much faster (80%) as seen in the results recorded in Table III. The second bud in serial order of development produced healthy and vigorous shoot while the shoots produced from third, fourth and fifth buds grew into shoots of smaller size. Hence, the second axillary bud was found suitable as an explant for multiplication. Further, increase in Kn levels of the culture media to 4 and 6 μM/l, declined the growth and development of the second axillary bud while axillary bud sprouted into four shoot-buds with callus at the base in response to 8 μM/l of Kn(Fig.5a).

Result of this factoral experiment proved that the second axillary bud developed into a single healthy shoot in presence of 2 μM/l of Kn. At 8 μM/l of Kn, the second axillary bud sprouted into four shoot-buds but with callus at the base

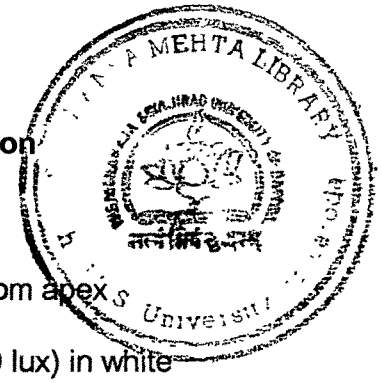


TABLE III : Effect of cytokinin/s on axillary bud proliferation

Medium : MS + Sucrose (3%) +Kn / BAP / Kn+BAP

Inoculum : A nodal segment with axillary bud (1.0-1.5 cm) from apex

Incubation : Four weeks at 25 ± 2°C in 16h photoperiod (1000 lux) in white fluorescent light

Sr. No.	Cytokinin/s levels (μM/1)		No. of shoot-buds induced/ explant	% response
	Kn	BAP		
1	o	o	-	-
2	1	-	1	70
3	2	-	1	80
4	4	-	1	50
5	6	-	1	50
6	8	-	4±0.15 + C	50
7	-	1	1	50
8	-	2	3±0.22	70
9	-	4	2±0.04	60
10	-	6	1	50
11	-	8	1	60
12	2	2	5±0.35	90
13	2	4	4±0.20	70
14	2	6	2±0.10	60
15	4	2	4±0.33	50
16	4	4	3±0.25	50
17	4	6	3±0.20	60
18	6	2	2±0.15	65
19	6	4	2±0.30	70
20	6	6	1	50
21	8	2	-	-
22	8	4	-	-
23	8	6	-	-

Mean of six replicates with standard deviation
C - Callus

Fig. 5 Second axillary bud cultured on MS+ Sucrose (3%)

a) + Kn (8 µM/l)

b) + BAP (2 µM/l)

c) + Kn (2 µM/l) + BAP (2 µM/l)

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

a

b

c

Fig. 5

and hence, they were unsuitable for multiplication of the species.

b) Effect of BAP

Nodal segments with axillary bud, second from the apex of a twig (1-1.5 cm) were collected, washed and surface sterilized as per the procedures described in Chapter II, Materials and Methods. Each bud was inoculated in Erlenmeyer flask (150 ml) containing MS basal medium (30 ml), sucrose (3%) and supplemented with BAP (0,1,2,4,6 and 8 µM/l) separately. Culture flasks were incubated as usual.

Results recorded (Table III) at the end of four weeks showed that the axillary bud continued its growth in response to 1µM/l of BAP.Increase in BAP level to 2 µM/l induced sprouting of the second axillary bud into 3±0.22 shoot-buds in 70% cultures. Out of these three buds, the original bud grew faster and attained a length of 8-9 cm as seen in Fig. 5b. Further increase in BAP level to 4 µM/l, not only reduced the number of sprouted buds to 2±0.04 but the percentage response was also

reduced to 60%. In addition, the length of the original shoot-bud was decreased resulting in dwarf rosette type of shoot-buds. Still further enhancement of BAP levels to 6 and 8µM/l, the axillary buds remained without sprouting.Thus, out of the BAP levels tested, 2µM/l was found to be superior because highest number of shoot-buds were induced from the axillary bud. Hence,a suitable combination of Kn with BAP was to be determined which would further increase the number of sprouted shoot-buds.

c) Effect of Kn in combination with BAP

Erlenmeyer flasks (150 ml) containing MS medium (30ml), with sucrose (3%)and Kn (2,4,6 and 8µM/l) in combination with BAP (2,4 and 6µM/l) were inoculated with second axillary bud, each after subjecting to surface sterilization procedures. Culture flasks were incubated as usual in culture room.

In results recorded (Table III) it was seen that in response to Kn with BAP each at 2µM/l , the axillary bud was

swollen within a week. At the end of four weeks, 5±0.35 shoot-buds (Fig.5c) in 90% of the cultures were produced. All these shoot-buds being strong, healthy and separable, could be used as propagules for multiplication. Each shoot-bud on separation and subculturing on fresh medium of the same composition produced a crop of four shoot-buds within four weeks. Thus, at the end of eight weeks culture period, from second axillary bud, about 20 healthy shoot-buds were produced. Keeping Kn level 2μM/l in combination with BAP (4μM/l) the axillary bud produced 4±0.20 shoot-buds in 70% of cultures. Further increase in BAP level to 6 μM/l resulted in a decline in the number of buds sprouted per explant to 2±0.10 in 60% cultures.

Increasing Kn level to 4μM/l in combination with BAP (2μM/l), the number of shoot-buds sprouted from the axillary bud were 4±0.33 in 50% cultures. Keeping Kn level constant (4μM/l) and increasing BAP level to 4 and 6μM/l, there occured reduction in number of shoot-buds sprouted to 3± 0.25 and 3±0.20 respectively. Thus, Kn (4μM/l) with BAP at the

levels tested, cultured axillary bud failed to enhance the number of shoot-buds.

Further increase in Kn level to 6μM/l with BAP (2 μM/l) resulted in a decline in the number of shoot-buds sprouted to 2±0.15, while in 4 and 6μM/l of BAP 2±0.30 and 1 shoot-buds were produced respectively.

At Kn (8μM/l) with BAP (2μM/l), the shoot-buds produced were fleshy with callus mass at the base of the explant. Keeping Kn level constant and increasing BAP level to 4 and 6μM/l, the axillary bud failed to sprout. Thus, Kn (8μM/l) in combination with BAP at the levels tested failed to induce sprouting of the cultured bud.

It was obvious that Kn with BAP each at 2μM/l, combination due to their synergistic effect produced maximum number of shoot-buds(5±0.35). Hence, this medium was designated as 'Multiplication Medium' for this species. In order to maintain the vitality of shoots produced, the rate of

multiplication was kept under control. In further experiments, this multiplication medium was employed.

Experiment 3 : Effect of auxin (IAA/NAA) on axillary bud proliferation

In this experiment, in multiplication medium auxin/s, *viz.*, Indole-3-acetic acid (IAA) or α-Napthalene acetic acid (NAA) was added to find out its effect on the increase in the rate of axillary bud proliferation.

MS multiplication medium (control) was supplemented with IAA/NAA at 0.5, 1 and 2μM/l levels. Surface sterilized nodal segment with axillary buds were inoculated inserting their lower portions in the media (30ml) in culture flasks. Six replicates per treatment were maintained in the culture room at 25±2°C in 16h photoperiod.

Results (Table IV) showed that axillary bud sprouted into 5±0.35 shoot-buds in 90% cultures in control (Fig.6a). But, addition of IAA at 0.5μM/l to the culture medium reduced

TABLE IV : Effect of auxin/s on axillary bud proliferation

Medium : Multiplication medium + IAA/NAA

Inoculum : An axillary bud

Incubation : Four weeks at 25 ± 2°C in 16h photoperiod (1000 lux) in white fluorescent light

Sr. No.	Auxin level (μM/1)	No. of buds induced/ explant	% response
1	Control - (Kn + BAP each at 2μM/l)	5±0.35	90
	IAA		
2	0.5	3±0.15 + C	60
3	1.0	4 ±0.15 + C	50
4	2.0	1+ C	40
	NAA		
5	0.5	1+ C	50
6	1.0	- do -	50
7	2.0	- do -	45

Mean of six replicates with standard deviation
C - Callus

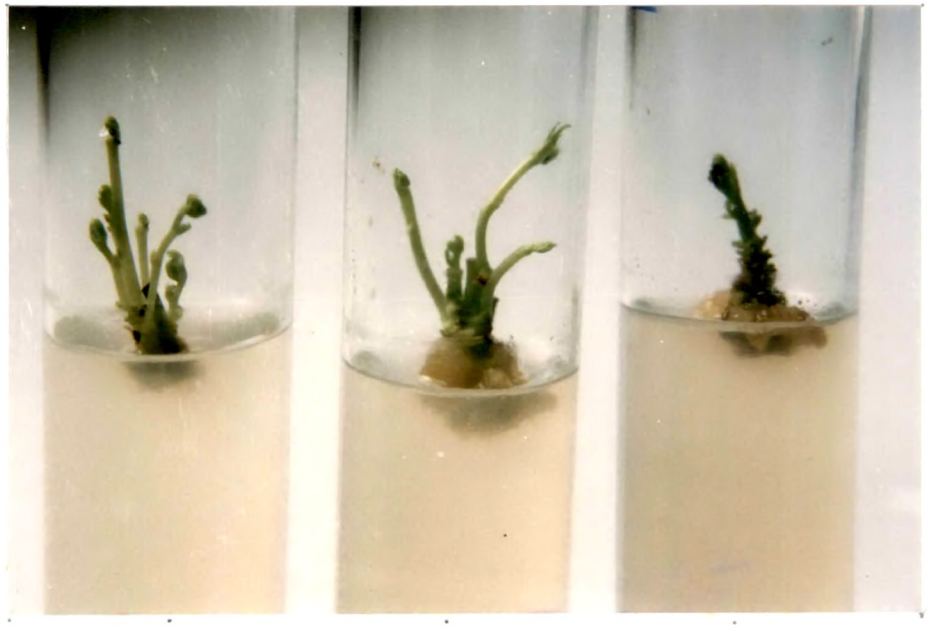
Fig.6 Second axillary buds cultured on MS + Sucrose (3%)

a) + Kn (2 µM/l) + BAP (2 µM/l) - Control

b) Control + 1 µM/l of IAA - Four buds with callus

c) Control + 1 µM/l of NAA - Original bud with callus

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



a b c

Fig. 6

proliferation rate of cultured bud to 3±0.15 shoot-buds. Further, callus was also developed at the base of the explant. Increase in IAA level to 1μM/l, the number of shoot-buds sprouted from the axillary bud increased to 4±0.15 with profuse callus production (Fig.6b). In presence of 2μM/l of IAA, the axillary bud failed to sprout and it grew into single shoot with large amount of callus at its base. Thus, it was evident that IAA at 0.5, 1 and 2μM/l failed to increase the rate of sprouting of the axillary bud.

In response to α-NAA at 0.5μM/l, axillary bud grew into a single shoot without sprouting. Increase in NAA to 1μM/l in the culture medium, the original bud continued its growth with callusing at the base (Fig.6c). Further increase in NAA level to 2μM/l, caused swelling of the axillary bud with profuse callus at the base.

Thus, it was evident that addition of IAA/NAA (0.5, 1 and 2μM/l) failed to enhance the rate of proliferation of axillary bud.

Experiment 4 : Selection of suitable culture medium for axillary bud sprouting

This experiment was conducted to find out a suitable culture medium supporting highest number of shoot-bud proliferation from the axillary bud . Five known synthetic culture media *viz.*, Murashige and Skoog (MS, 1962); Wood and Braun (WB, 1961); Gamborg's (B_5,1968); Schenk and Hilderbrandt, (SH, 1972) and Lloyd and Mc Cown (LM,1981) with their compositions as given in Table I (a-e), Chapter II were used to find out a suitable culture medium amongst them supporting maximum number of shoot-bud proliferation from the axillary bud. All these media were supplemented with sucrose (3%) and Kn with BAP each at 2µM/l, as present in multiplication medium (Expt.2). Surface sterilized second axillary bud was inoculated on above said media (30 ml).Culture flasks were incubated as per the experimental procedures described earlier.

Results of Fig.7 showed that 5±0.05 shoot-buds sprouted from single axillary bud on MS medium at the end of

Fig. 7 Axillary buds cultured on sucrose (3%) + (Kn + BAP, 2 µM/l each) +

a) Murashige and Skoog medium
b) Wood and Braun medium
c) Gamborg's B_5 medium
d) Schenk and Hilderbrandt medium
e) Lloyd and Mc Cown medium

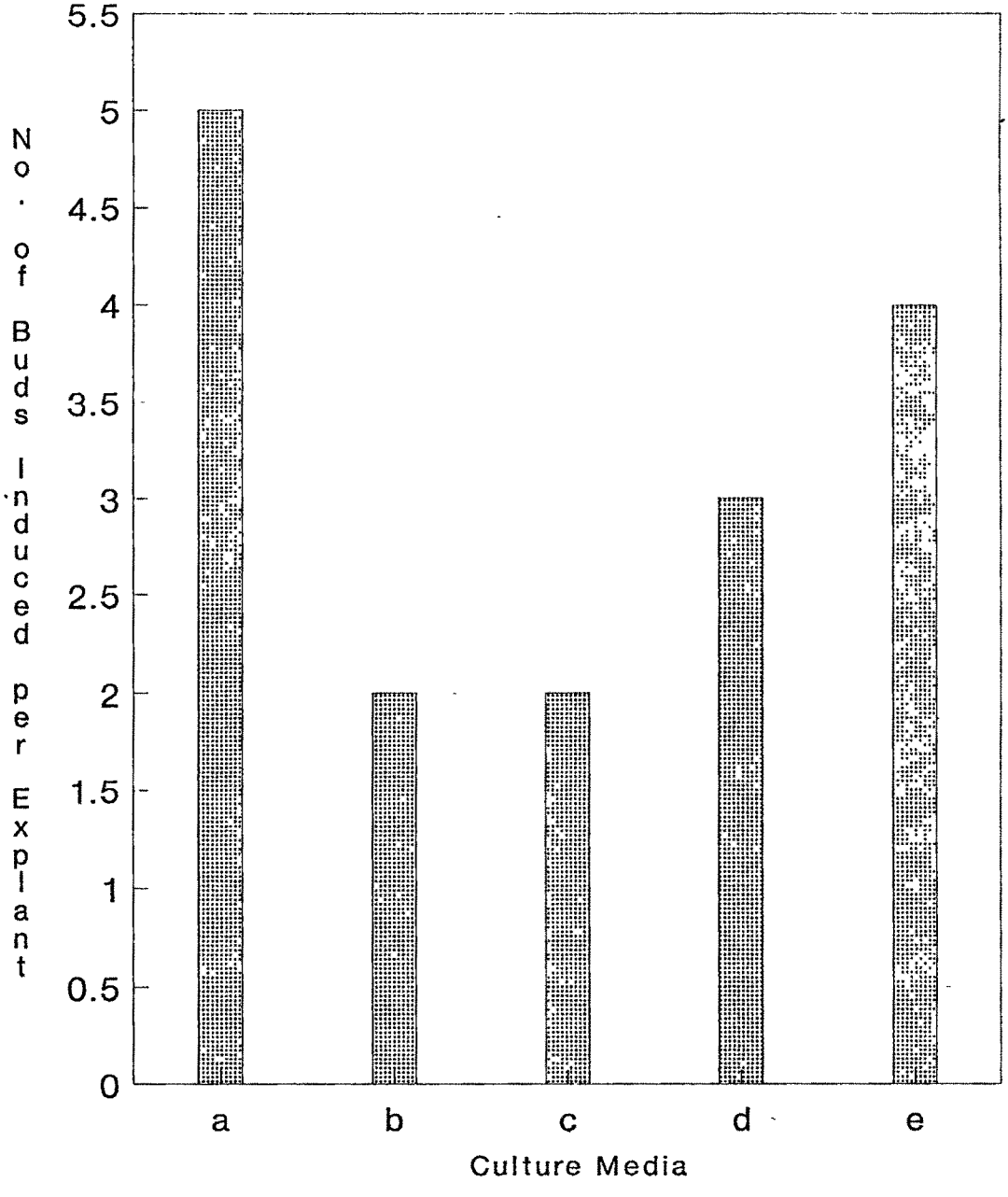

5.5
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
No. of Buds Induced per Explant
a
b
c
d
e
Culture Media

Fig. 7

four weeks. All five shoot-buds produced were healthy and were easily separable. Axillary buds cultured on WB medium produced 2±0.15 shoot-buds while on B_5 medium 2±0.05 shoot-buds with profuse callus at the base were produced. Axillary buds cultured on SH media sprouted into dwarf and succulent 3±0.20 shoot-buds while healthy, separable 4±0.50 shoot-buds were produce on LM media.

Comparing the number of shoot-buds produced on various media it was evident that MS medium with its supplements supported highest number of shoot-bud (5±0.05) sprouting from a single axillary bud. Hence, MS medium was selected as a suitable culture medium.

Factoral experiments were conducted to find out the influence of

a) Inorganic nitrogen,

b) Vitamins and

c) Inositol

as present in MS medium on the rate of sprouting of axillary buds.

a) Influence of inorganic nitrogen

In order to find out the effect of variation in the inorganic nitrogen levels of the MS medium, they were adjusted as its absence, half dose, standard and double doses keeping 840mg/l as the standard dose of inorganic nitrogen. The ionic balance of the culture media was adjusted using KCl.

Surface sterilized axillary buds were inoculated on the above culture media (30 ml). Six replicates per treatment were incubated in culture room as usual.

Axillary buds cultured on MS media in absence of inorganic nitrogen continued their growth for a while but all of them died at the end of the experimental period. Inorganic nitrogen at half its level of the standard dose in the medium induced three shoot-buds per explant but they were chlorotic. However, in the standard dose of inorganic nitrogen in culture

media five healthy shoot-buds sprouted from the axillary bud at the end of four weeks (Fig.8). Further, doubling the level of inorganic nitrogen in the medium also failed to induce sprouting of the cultured bud.

It was proved that the level of inorganic nitrogen as present in MS medium was at its optimal level for axillary bud sprouting.

b) Influence of vitamins

In this factoral experiment, MS multiplication medium (30 ml) in absence, half dose, standard and double doses of vitamins as present in original media were altered to find out their influence on axillary bud sprouting. Culture conditions were maintained the same.

Results of Fig.8 indicate that in absence of vitamins the cultured bud failed to sprout. However, in multiplication media containing half strength vitamins, 2.5 shoot-buds sprouted from the cultured bud while at its standard dose, 5

Fig. 8 Axillary buds cultured on MS medium in

a) Absence of inorganic nitrogen/ vitamins/inositol
b) Half dose of " " " "
c) Standard dose of " " "
d) Double dose of " " "

containing sucrose (3%) and (Kn + BAP, 2 µM/l each)

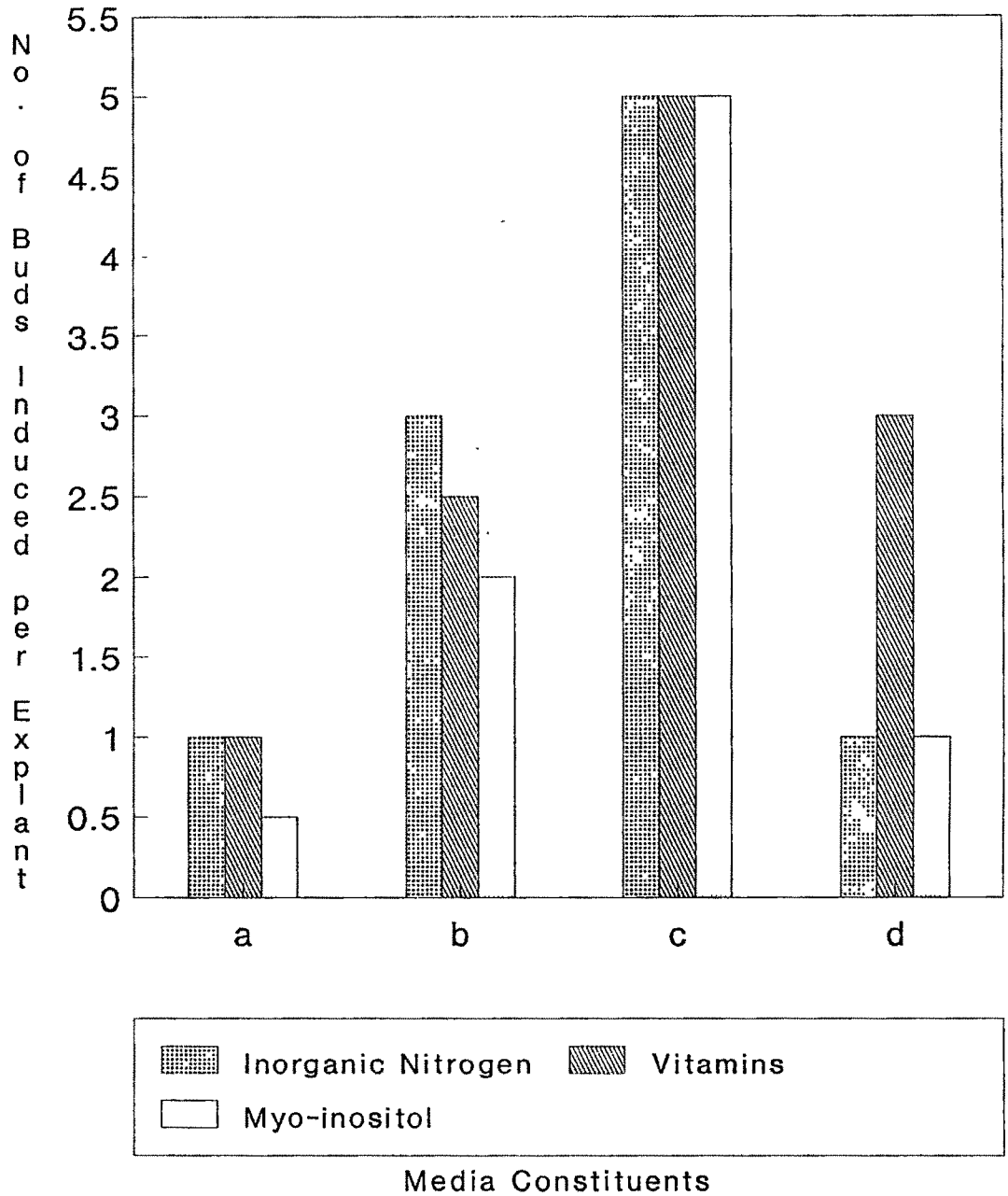

No. of Buds Induced per Explant
5.5
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
a
b
c
d
Inorganic Nitrogen
Vitamins
Myo-inositol
Media Constituents

Fig. 8

robust shoot-buds were produced. Further, doubling the dose of vitamins in the medium, the rate of proliferation of axillary buds declined.

Hence, standard dose of vitamins as present in MS medium was at its optimum level for axillary bud sprouting.

c) Influence of inositol

MS media (30 ml) in complete absence/half dose/standard/double dose of inositol were altered to evaluate their influence on axillary bud sprouting. Culture flasks were incubated following the usual procedures as described in Chapter II, Materials and Methods.

Results after four weeks (Fig.8) showed that in absence of inositol the axillary buds were swollen without sprouting. However, inositol at its half dose as present in media supported proliferation of axillary bud into two shoot-buds with callus at its base. At the standard dose of inositol five healthy shoot-buds were produced from the cultured bud.

Further, doubling the inositol level, resulted in failure of the axillary bud to sprout.

It was evident from this experiment that 100 mg/l of inositol as present in MS medium was the optimal level required for maximum proliferation of axillary bud.

Experiment 5 : Effect of various carbohydrates on axillary bud sprouting

The carbohydrates added to the culture media serve as a source of energy required for the growth and development of the cultured explant. In the present experiment surface sterilized nodal explants with axillary buds were inoculated to MS multiplication medium (30ml) supplemented with 3% monosaccharides *viz.*, glucose, fructose, disaccharide *viz.*, sucrose; polysaccharides *viz.*, mannitol and starch separately to find out their effects on the rate of sprouting of the cultured buds. Culture flasks were maintained at 25±2°C in 16h photoperiod (1000 lux).

It was noticed (Table V) that in presence of glucose and fructose second axillary bud sprouted into 2±0.25 and 2±0.80 buds respectively (Fig.9 a&b). Axillary bud cultured on sucrose sprouted into 5±0.60 shoot-buds (Fig.9c).In presence of mannitol the axillary bud continued growth without its sprouting while in presence of starch the growth of the original axillary bud was arrested (100%).

Hence, 5±0.60 shoot-buds, being the highest number, were produced in presence of sucrose, proving its superiority over other carbohydrates.

The optimal level of sucrose, to induce highest number of shoot-bud sprouting was determined in this experiment. MS medium (30 ml) containing Kn+BAP each at 2µM/l was supplemented with sucrose (0,1,2,3 and 4%)and inoculated with surface sterilized axillary buds. Culture flasks were incubated as usual.

TABLE V : Effect of various carbohydrates on axillary bud proliferation

Medium : Multiplication medium + 3%(Glucose / Fructose / Sucrose/ Mannitol / Starch)

Inoculum : An axillary bud

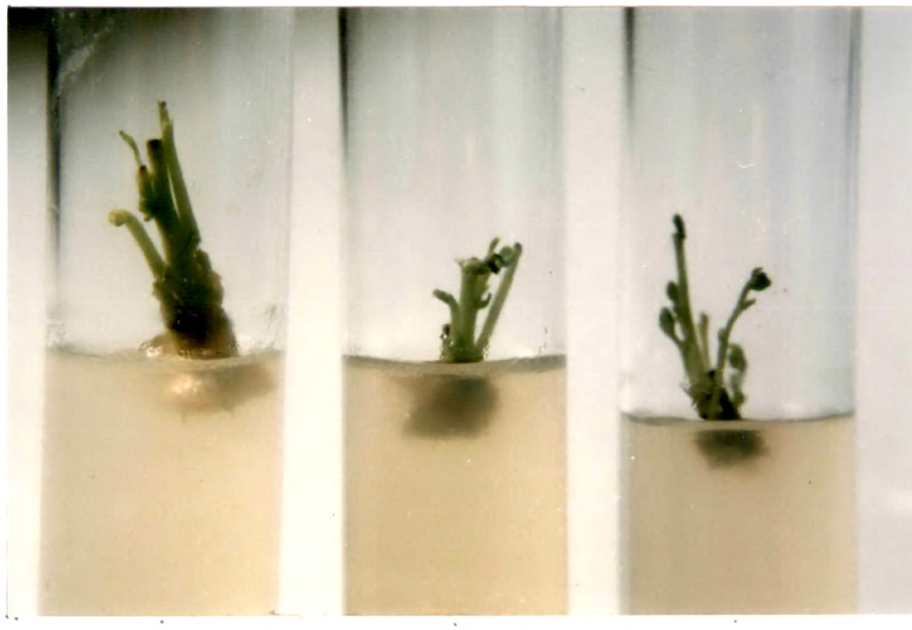
Incubation : Four weeks at 25±2°C in 16h photoperiod (1000 lux)

Sr. No.	Carbohydrates (3%)	Number of buds induced/explant	% response
1	Glucose	2±0.25	60
2	Fructose	2±0.80	60
3	Sucrose	5±0.60	90
4	Mannitol	1	70
5	Starch	-	-

Mean of six replicates with standard deviation

Fig.9 Sprouting of second axillary bud cultured on multiplication medium containing 3% of

a) Glucose
b) Fructose
c) Sucrose



a b c

Fig. 9

Results recorded after four weeks culture period showed that in absence of sucrose, the cultured axillary bud turned black and died while in presence of 1% and 2% of sucrose, the number of shoot-buds sprouted were 1 and 4 respectively as seen in the results of Fig.10. Highest, 5 shoot-buds were produced from single axillary bud in presence of 3% sucrose. Further increase in sucrose to 4% declined the rate of axillary bud sprouting to 2.

Thus, sucrose at 3% proved to be the optimal level for axillary bud sprouting.

Experiment 6 : Histological studies during axillary bud sprouting

In the present experiment histological changes in the tissues of the axillary bud in response to cytokinins (Kn+BAP each at 2µM/l), during its sprouting were studied. Serial sections of the concerned tissues were prepared and stained according to the procedures described in Chapter II, Materials and Methods.

Fig.10 Effect of sucrose
a) 0%
b) 1%
c) 2%
d) 3%
e) 4%
on axillary buds sprouting

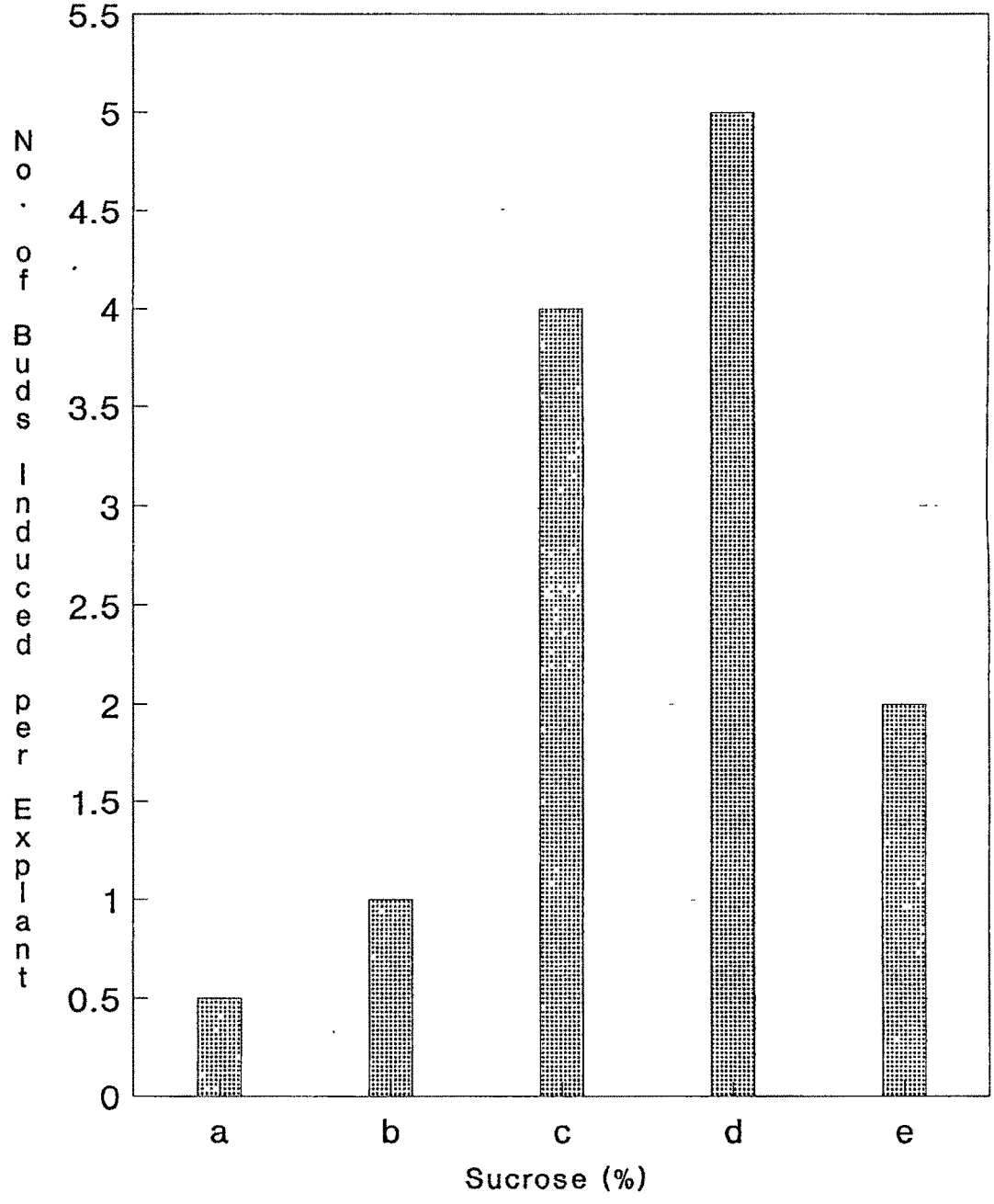

No. of Buds Induced per Explant
5.5
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
a
b
c
d
e
Sucrose (%)

Fig. 10

In the leaf axil, surrounding the axillary bud (Fig.11,o), quiescent meristems (q) were located. In response to cytokinin treatment, cell division takes place in the cells of quiescent meristem. As a result, fresh bud (b) initiated from them. This process occured during sprouting of the axillary bud.

Experiment 7 : Ideal period during the year for optimal axillary bud sprouting

In order to find out the ideal period - month of the year when maximum number of shoot-buds would be produced from a single axillary bud cultured on multiplication medium, this experiment was conducted.

Axillary buds were inoculated on multiplication media (30ml) and culture flasks were incubated in a culture room at 25±2°C in 16h photoperiod (1000 lux). Experiment was conducted after every two months *viz.*, (a) Jan.-Feb. (b) March-April (c) May-June (d) July-Aug. (e) Sept.-Oct. (f) Nov.-Dec. Results after four weeks culture period were recorded in

Fig. 11 L.S. of nodal segment with axillary bud showing initiation of bud (b) in quiescent meristems (q) (10X x 16X).

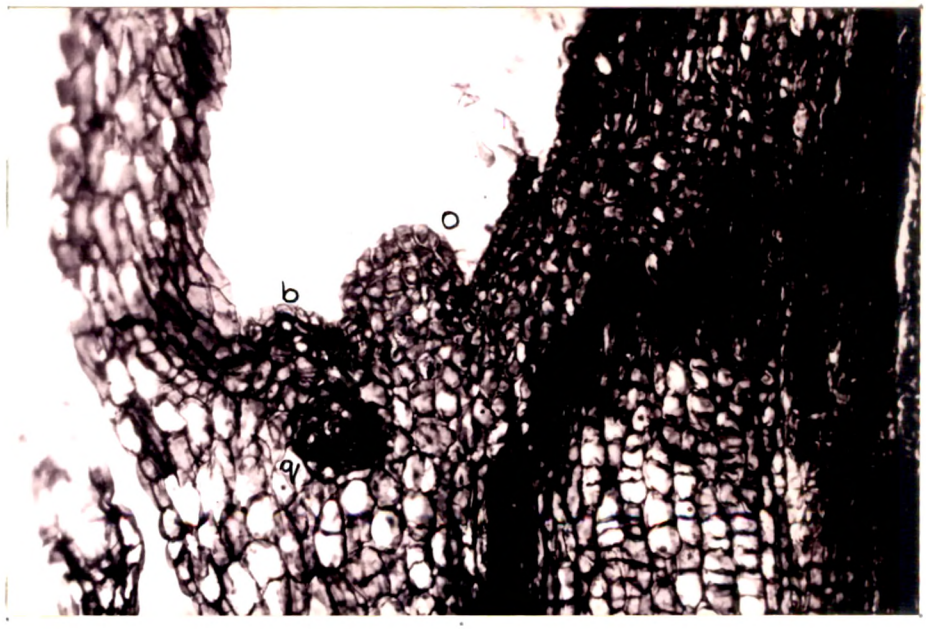


Fig.11

Table VI. The maximum-minimum temperatures and relative humidity during these months were also recorded.

Axillary buds when cultured in the months of Jan.-Feb. on MS multiplication medium failed to sprout. The average maximum and minimum temperatures during these months were 29°C and 14°C respectively with relative humidity 45 as seen in Table VI. In the months of March and April i.e. spring season, when the level of endogenous growth regulator rises, the cultured axillary bud sprouted into five shoot-buds within four weeks. At this juncture, the maximum and minimum temperature were 36°C and 21°C respectively with relative humidity as 46. With the rise in temperature during the months of May-June there was a decline in the number of buds sprouted to three. Further, in the months of July-Aug., a fall in temperature and rise in relative humidity resulted in growth of a single bud per cultured explant (Table VI). Still further, Sept.-Oct. being autumn months when new sprouts appeared, axillary bud sprouted into four buds per explant. Subsequently, in the months of Nov.-Dec. there was no sprouting of fresh axillary

TABLE VI : Ideal month for axillary bud sprouting

Medium : Multiplication medium

Inoculum : An axillary bud

Incubation : Four weeks at 25 ± 2°C in 16h photoperiod (1000 lux) in white fluorescent light

Months	Average Temperature °C		Avg. Relative Humidity	No. of shoot-buds/ explant
	Max.	Min.		
Jan. - Feb.	29	14	45	-
Mar. - Apr.	36	21	46	5
May - June	39	28	42	3
July - Aug.	33	24	81	1
Sept. - Oct.	34	22	55	4
Nov. - Dec.	31	20	40	-

Mean of six replicates

buds on the plant. Hence, when the experiment was conducted from the existing buds, they failed to sprout.

Results of this experiment clearly indicate that axillary buds sprouted on 'elite' *D.sissoo* in the months of March-April, when cultured on multiplication medium produced highest number of shoot-buds. Each of the shoot-bud after isolation could be used as vegetative-propagule for the production of a healthy plant.

II. PLANT REGENERATION FROM CALLUS CULTURES

This section describes successful establishment of callus tissues from stem and leaflet segments of 'elite' *D.sissoo* plant. Murashige and Skoog (1962) media containing sucrose (2%) and supplemented with phytohormones as per the requirements of the experiment were used to carry out experimental work on the callus tissues, their sufficient quantities were required, hence the nutritional and hormonal levels supporting their highest biomass production were standardised. Further, the biosynthetic potential of callus

tissues for quercetin synthesis/ accumulation was investigated. Also the morphogenic potential of callus tissues for plant regeneration was examined.

Experiment 8 : Callus induction from stem/leaflet pieces of 'elite' *D.sissoo plant*

Healthy stem and leaflet pieces from 'elite' plant of *D.sissoo* were collected in Erlenmeyer flask containing distilled water. After surface sterilization they were cut into desired sizes and these explants were inoculated in Erlenmeyer flask containing MS medium (30ml) with sucrose (2%) supplemented with Kn/BAP (0,0.5,1 and 2µM/l) alone and in combination with IAA/NAA/2,4-D in various concentrations (0,0.5,1,2 and 4µM/l). Six replicates per treatment were incubated at 25±2°C in 16h photoperiod.

Results recorded in Table VII indicate that stem explants cultured on basal MS medium turned black within one week culture period. In presence of Kn (0.5µM/1), the explants remained green during first week but turned black later on.

TABLE VII : Callus induction from stem segments/leaflets of *D. sissoo* Roxb.

Medium : MS + Sucrose (2%) + BAP/Kn / (Kn + IAA/NAA/2,4-D)

Incubation : Four weeks at 25 ± 2°C in 16 h photoperiod (1000 lux) in white fluorescent light

Sr. No.	Auxin (μ M/1)	BAP (0.5 - 2 μM/l) / Kn (μ M/1)	Stem segments				Leaflets			
			–				–			
			0.0	0.5	1.0	2.0	0.0	0.5	1.0	2.0
1	Control		-	-	-	-	-	-	-	-
	IAA									
2	0.0		-	-	-	-	-	-	-	-
3	0.5		-	-	-	-	-	-	-	
4	1.0		-	-	+	+	-	-	+	+
5	2.0		-	+	++	++	-	+	++	++
6	4.0		-	+	+	+	-	+	+	+
	NAA									
7	0.5		-	-	-	-	-	-	-	-
8	1.0		-	-	+	+	-	+	+	+
9	2.0		-	+	+	+	-	+	++	++
10	4.0		-	+	+	+	-	+	+	+
	2, 4-D									
11	0.5		-	+	+	+	-	+	+	+
12	1.0		-	+	++	++	-	+	++	++
13	2.0		-	++	++++	++	-	++	++++	++
14	4.0		-	++	++	++	-	+	++	++

Mean of six replicates
- No response
+ Amount of callus induced

Increase in Kn level to 1/2μM/l in the culture media kept the explants green for two weeks and then turned black. When BAP at 0.5,1 and 2μM/l was tested, explants remained green for one week without callus initiation. This proved that Kn or BAP alone failed to induce callus from stem pieces.

Leaflet explants, when cultured on MS basal medium in absence of Kn failed to show any response and subsequently died. In Kn (0.5, 1 and 2μM/l) supplemented media cultured leaflets showed a loss of chlorophyll and increase in its size without callus induction. In media containing BAP (0.5,1 and 2μM/l), there was curling of leaflet pieces without any sign of callus initiation.

Hence, among the cytokinin/s (Kn/ BAP) tested, Kn was chosen and subsequently it was used in combination with auxins (IAA/NAA/2,4-D) in order to test their ability for callus induction from explants.

Stem explants in response to Kn (0.5μM/l) with IAA (0.5/1μM/l) failed to show callus initiation. Keeping Kn level constant, when IAA was increased to 2/4μM/l, callus was induced within four weeks. Increasing Kn level to 1/2μM/l with IAA (0.5μM/l), stem pieces failed to induce callus. At Kn (1/2μM/l) with IAA (1μM/l), stem explants induced slight callus from their cut ends in four weeks. Keeping Kn (1/2μM/l) constant and increasing IAA level to 2μM/l resulted in induction of callus after two weeks in cultured stem explants which in presence of IAA (4μM/l) showed a decline in callus induction .

Leaflet explant cultured on Kn (0.5μM/l) in combination with IAA (0.5/1μM/l) resulted in growth and subsequent curling of leaflets which failed to show callus induction. Keeping Kn level constant and increasing IAA level to 2/4μM/l, slight callus initiation occured from the margins of leaflets at the end of four weeks. Increase in Kn level to 1/2μM/l with IAA(1μM/l) resulted in slight callus initiation in four weeks while leaflet pieces in presence of 2μM/l of IAA resulted in initiation of callus at a faster rate requiring about two weeks for

the same. However, this callus was compact. At Kn (1/2μM/l) with IAA (4μM/l) callus was induced in three weeks.

Stem explants when cultured on Kn (0.5μM/l) with NAA (0.5/1μM/l) failed to show callus induction while at 2/4μM/l of NAA, slight callus was induced in four weeks. At Kn (1/2μM/l), in combination with NAA (1/2/4μM/l),light yellow coloured callus was induced from cut ends of stem explant in four weeks.

Leaflet pieces when cultured on Kn (0.5/1/2μM/l) with NAA (0.5μM/l) failed to show callus induction. Keeping Kn (0.5μM/l) level constant and varying NAA level from 1/2/4μM/l, explant showed slight callus initiation. Increasing Kn level to 1/2μM/l with NAA (1μM/l) callus induction occured in four weeks while at 2μM/l of NAA callus was induced in two weeks. Further increase in NAA level to 4μM/l resulted in induction of callus at a slower rate (Table VII).

Stem explants in presence of Kn (0.5μM/l) with 2,4-D (0.5/1μM/l) induced callus in four weeks. Increase in Kn level to 1/2μM/l in combination with 2,4-D (1μM/l), cultured stem explant showed callus induction in two weeks culture period . However, in presence of Kn (0.5μM/l) with 2,4-D (2/4μM/l),stem explants induced callus in two weeks. In presence of Kn (1μM/l) with 2,4-D (2μM/l) callus was induced in one week. This callus was cream coloured, friable in nature and originiated from cut ends of stem explant (Fig.12a). At Kn (1/2μM/l) in combination with 2,4-D (4μM/l), stem pieces showed callus initiation in three weeks.

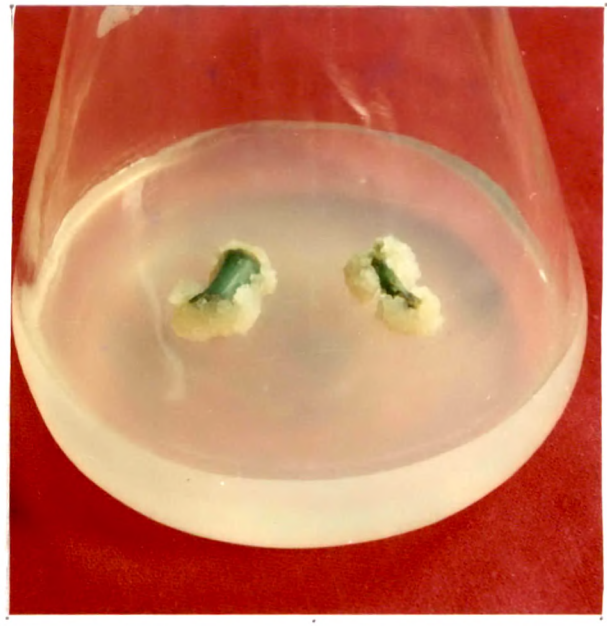
Leaflet explants in response to Kn(0.5/1/2μM/l) in combination with 2,4-D (0.5μM/l) showed increase in their size with slight callus initiation in four weeks. With increase in 2,4-D level to 1/2μM/l, callus was induced earlier (Table VII) maximum being at Kn (1μM/l) with 2,4-D (2μM/l) (Fig.12b). Further increase in 2,4-D level to 4μM/l resulted in callus requiring more time for its induction.

Fig.12 Callus induced from

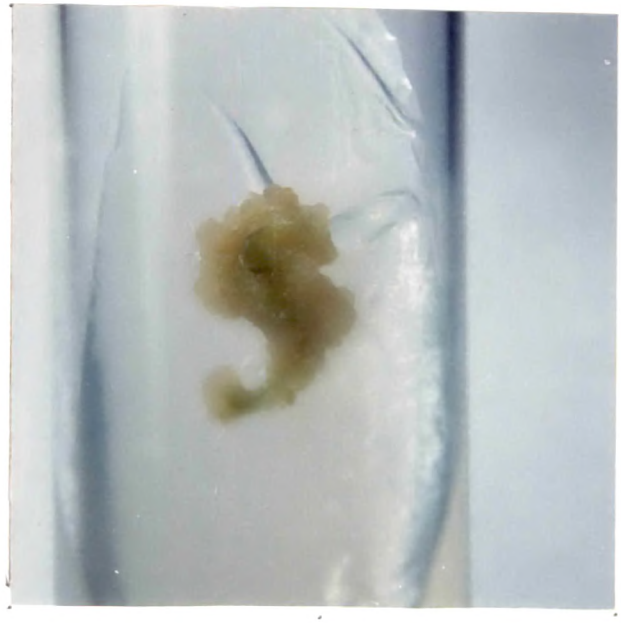
a) Stem pieces

b) Margins of leaflet

Culture period : Four weeks



a



b

Fig.12

Thus, Kn (1μM/l) in combination with 2,4-D (2μM/l) proved ideal combination for callus initiation from the stem as well as leaflet explants.

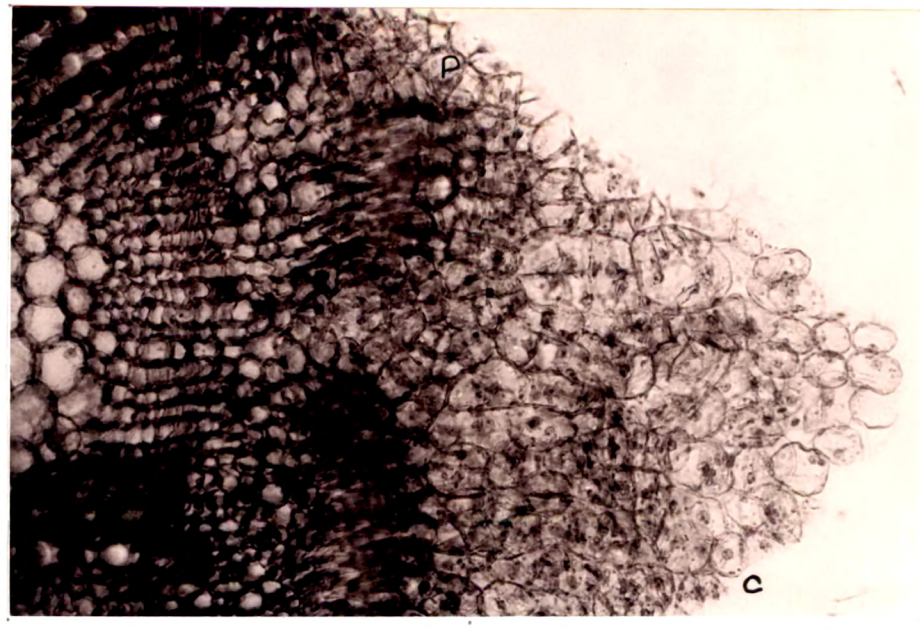
Histological studies during callus initiation revealed that the epidermal cells of the stem burst open in response to the treatment given and the cells of underlying parenchymatous cortical tissue (P), assumed division. This resulted in callus (C) initiation from the parenchymatous cortex (Fig.13a). The leaflets in response to Kn (1μM/l) and 2,4-D (2μM/l) were swollen , as a result the epidermal cells were disorganized. Subsequently, from the spongy parenchymatous cells (Sp), callus was initiated(Fig. 13b). These callus cells were rounded and with thin walls.

Experiment 9 : Optimal biomass production of callus cultures

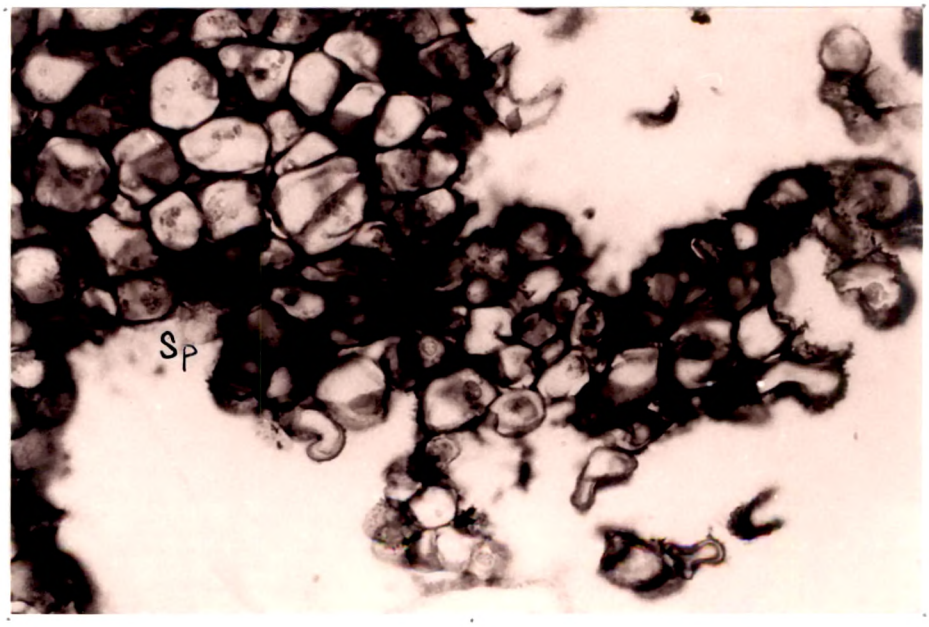
In the present experiment, the nutritional and hormonal levels for the optimal biomass production of stem and leaflet callus cultures were standardised.

Fig.13 a) T.S of stem showing callus induction (c) from parenchyma cells of the cortex (p) (10 x 16 X)

b) T.S. of leaflet showing callus induction (c) from spongy parenchyma (sp) (10 x 25X)



a



b

Fig.13

a) Effect of various carbohydrates

This experiment was conducted to find out the effect of various carbohydrates on callus growth. MS media (30ml) containing Kn (1μM/l), 2,4-D (2μM/l) and supplemented with glucose/sucrose/mannitol (2%) were inoculated with stem/ leaflet callus tissues (fr. wt.,300±20 mg and dry wt., 20±01 mg).

Results in Fig.14 indicate that stem callus cultured on glucose supplemented media supported its growth, with the biomass value reaching upto 2645±30 mg and 175±05 mg in terms of fresh and dry weights (a) at the end of four weeks. These callus tissues were hard and compact. In sucrose containing media callus growth was much faster and the biomass values were 3450±25 mg, fr.wt. and 225±05 mg, dry wt.(b). Further, in mannitol supplemented media, poor growth of the callus tissues occured(c).

Leaflet callus on glucose supplemented media acquired fresh weight, 2800±45mg and dry weight, 180±02mg.

Fig.14 Growth of stem / leaflet callus tissues on MS medium containing Kn (1μM /l), 2, 4-D (2μM /l) and supplemented with (2%)

a) Glucose
b) Sucrose
c) Mannitol

Culture period : Four weeks

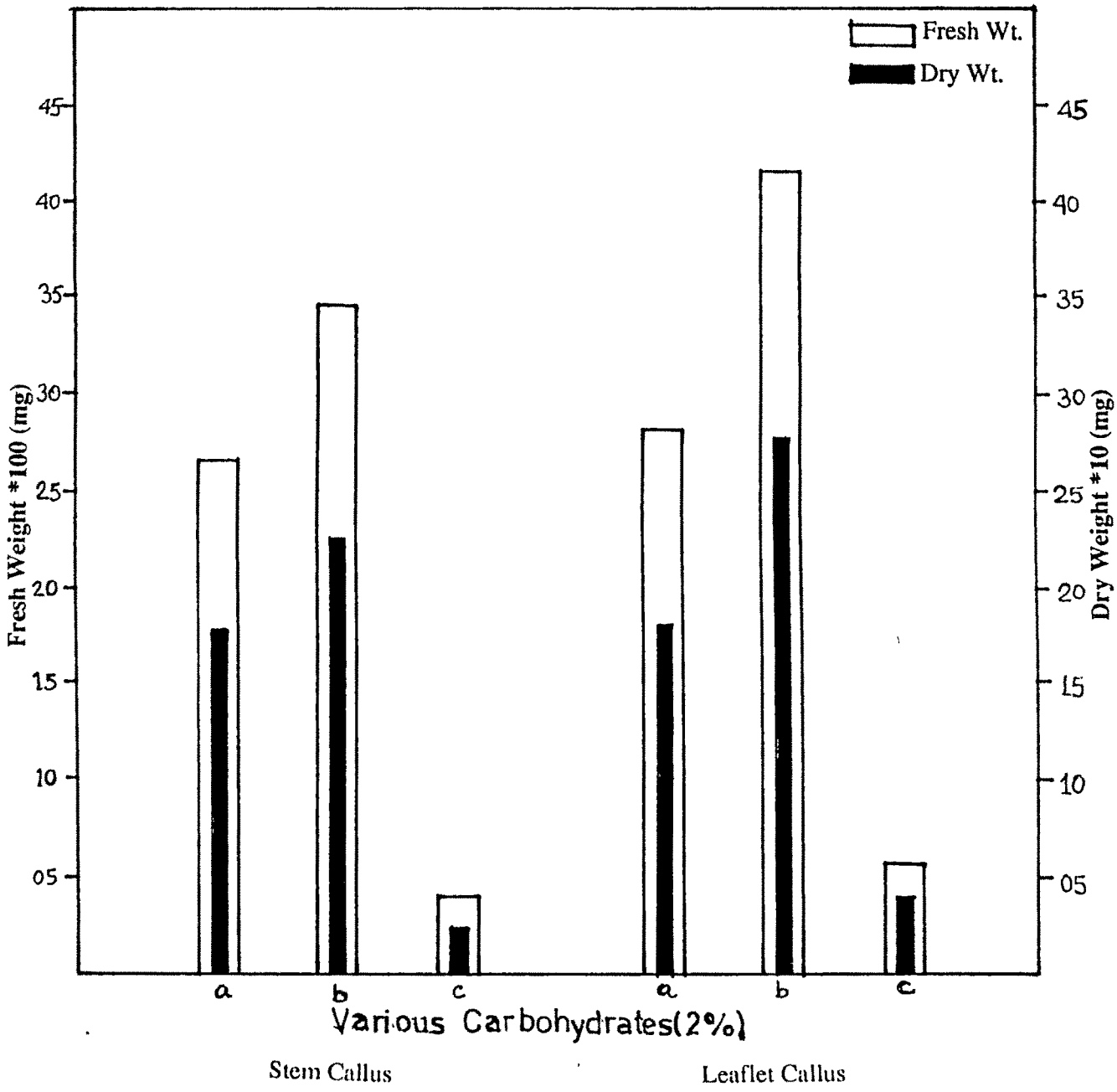

Fresh Wt.
Dry Wt.
Fresh Weight *100 (mg)
Dry Weight *10 (mg)
45
40
35
30
25
20
15
10
05
a
b
c
a
b
c
Various Carbohydrates(2%)
Stem Callus
Leaflet Callus

Fig.14

Faster growth of callus tissues was recorded when they were cultured on sucrose with biomass value reaching to 4150±65 mg and 275±02 mg in terms of fresh and dry weights while in mannitol, slight growth of callus was observed (Fig.14).

Results showed that sucrose supported highest growth of stem and leaflet callus tissues thereby proving the superiority of sucrose over other carbohydrates. Still, the level of sucrose required for optimal biomass of callus cultures remained to be determined.

Hence, MS medium (30 ml) containing Kn (1µM/l) in combination with 2,4-D (2µM/l) was supplemented with sucrose at various levels (0,1,2,3 and 4%) and stem/ eaflet callus tissues were transferred to them (300±20 mg, fr.wt. and 20±02 mg, dry wt.). Culture flasks were incubated as per the standard procedures.

Results presented in Fig.15 indicate that stem callus on medium without sucrose recorded very poor growth, as its

biomass value was 405±02 mg, fr. wt. and 20±01 mg, dry wt. at the end of four weeks period. Increase in sucrose level to 1%, stem callus recorded 1800±10 mg and 80±05 mg as fresh and dry weights respectively. Further, increase in sucrose level to 2% recorded two fold increase in biomass values, being 3500±20 mg and 230±05 mg in terms of fresh and dry weights respectively. Still further, increase in sucrose level to 3% and 4% resulted in a decline in fresh and dry weight values as recorded in Fig.15.

Leaflet callus when cultured on the medium lacking sucrose recorded 465±10 mg and 30.5±05 mg in terms of fresh and dry weights. Increase in sucrose level to 2% recorded optimal biomass production, being 4100±40 mg, fresh weight and 270±02 mg, dry weight. Decline in biomass values was recorded at 3% and 4% sucrose levels (Fig.15).

Hence, it was evident that sucrose at 2% was optimal for biomass production of both the callus tissues.

Fig.15 Growth of stem / leaflet callus tissues on

MS + Kn (1μM /l) + 2, 4-D (2μM /l) + Sucrose (0, 1, 2, 3 and 4%)

Culture period : four weeks

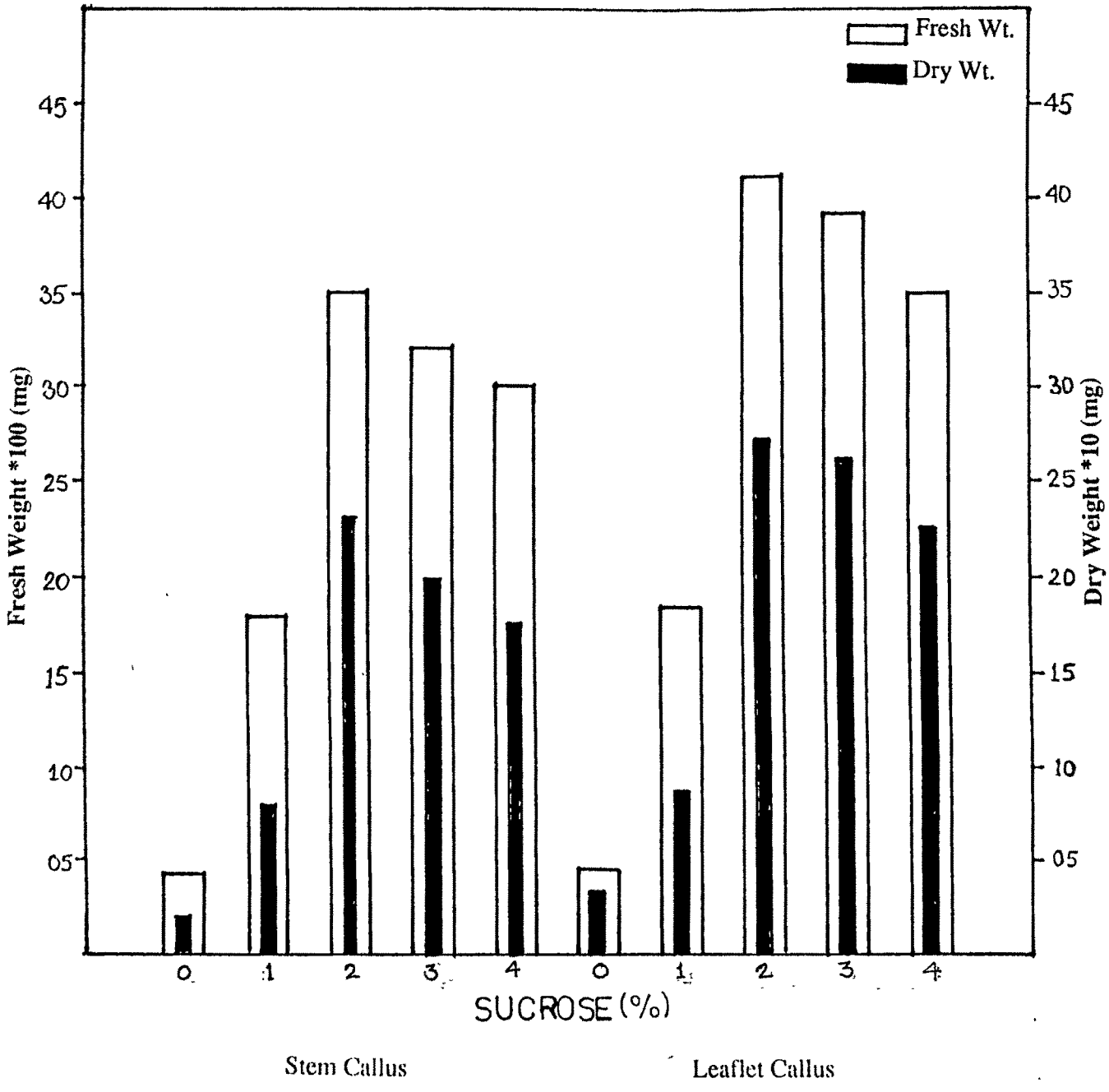

Fresh Wt.
Dry Wt.
Fresh Weight *100 (mg)
Dry Weight *10 (mg)
45
40
35
30
25
20
15
10
05
0
1
2
3
4
SUCROSE (%)
Stem Callus
Leaflet Callus

Fig.15

b) Effect of Kn levels

Healthy, fast growing callus cultures of stem/ leaflet (300±20 mg, fr. wt. and 20±02 mg, dry wt.) were inoculated on MS medium (30ml) containing sucrose (2%), 2,4-D (2µM/l) and supplemented with Kn at various levels (0,0.5,1&2µM/l). Culture flasks were incubated as per the procedures described in Chapter II, Materials and Methods.

It was observed that stem callus cultured on medium without Kn registered slight biomass production in terms of fresh and dry weights, their values being 600±20 mg and 40±05mg. With the incorporation of Kn (0.5µM/l) in the culture medium there was considerable enhancement in stem callus biomass production, reaching to 3125±50 mg and 220±06 mg by fresh and dry weights. However, at 1µM/l of Kn, the biomass production of the callus tissues reached to its maximum, being 3400±10 mg, fr. wt. and 190±02 mg, dry wt. Further increase in Kn level to 2µM/l resulted in a decline in biomass values (Fig.16).

Fig.16 Growth of stem / leaflet calllus tissues on

MS + Sucrose (2%) + 2, 4-D (2 μM /l) + Kn (0, 0.5, 1 and 2μM /l)

Culture period : Four weeks

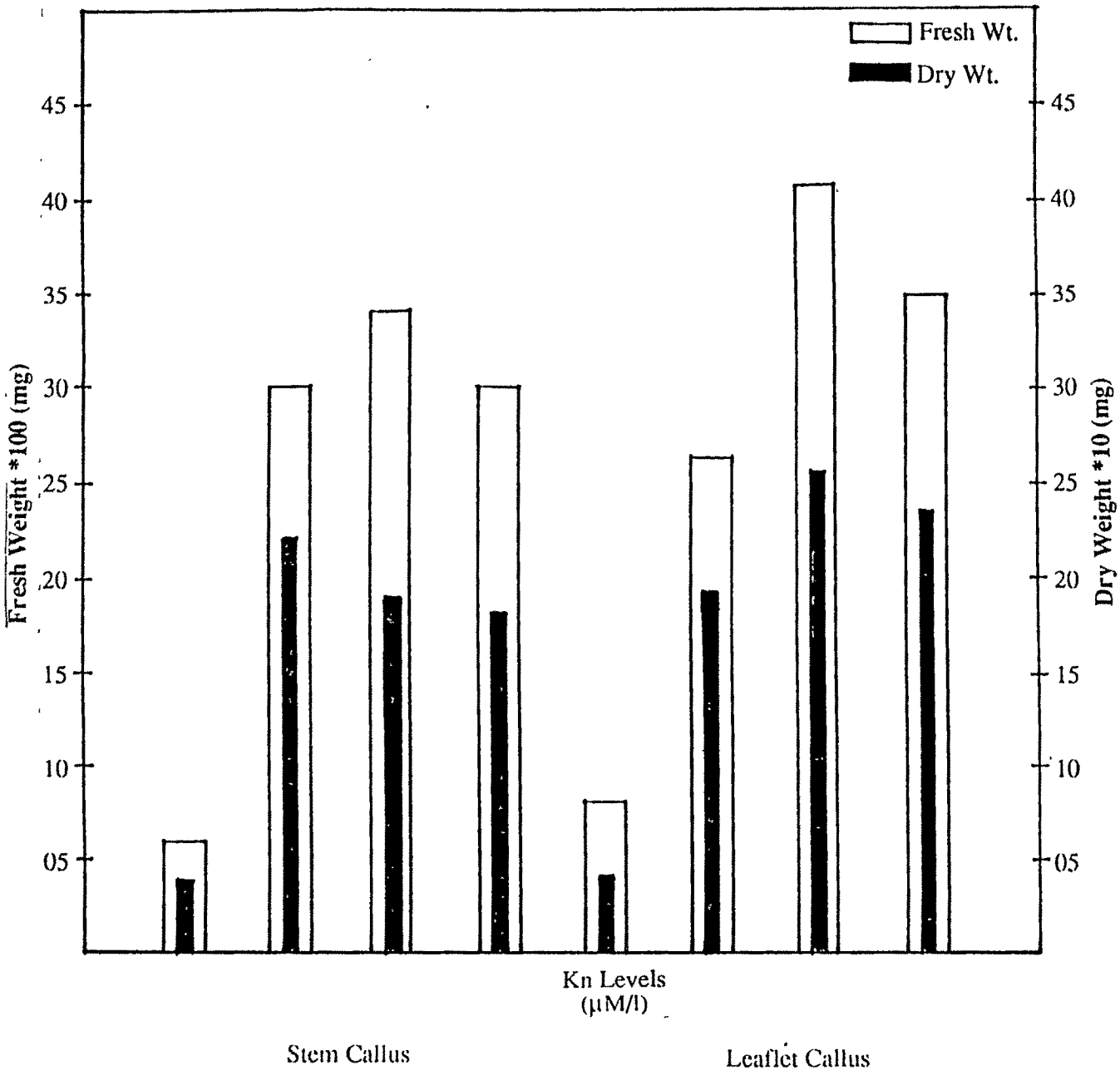

Fresh Wt.
Dry Wt.
45
40
35
30
25
20
15
10
05
Fresh Weight *100 (mg)
Dry Weight *10 (mg)
Kn Levels
(µM/l)
Stem Callus
Leaflet Callus

Fig.16

In the leaflet callus, in absence of Kn there was slight biomass production as these values were 750±20 mg and 40±02 mg by fresh and dry weights at the end of four week culture period. Addition of Kn in the culture medium at 0.5µM/l level the biomass production was enhanced to 2800±10 mg and 185±02 mg in terms of fresh and dry weights respectively. Callus tissues produced optimal biomass (fr.wt., 4050±25 mg and dry wt. 260±05 mg) when the Kn level was 1µM/l. Further, increase in Kn level to 2µM/l did not support the callus growth as their biomass values declined (Fig.16).

Hence, Kn at 1µM/l was found to be the optimal level for biomass production of stem and leaflet callus tissues.

c) Effect of 2,4-D levels

Stem and leaflet callus tissues, 300±20 mg and 20±02 mg by fresh and dry weights were transferred to Erlenmeyer flasks containing Kn (1µM/l) and supplemented with 2,4-D at various levels (0,0.5,1,2 and 4µM/l).

Results depicted in Fig.17 indicate that stem callus cultured in absence of 2,4-D recorded 620±03 mg and 40±02 mg of fresh and dry weights respectively. Addition of 2,4-D at 0.5μM/l produced considerable growth of callus tissues and their biomass values reached to 1500±20 mg and 100±02 mg by fresh and dry weights at the end of four weeks culture period. Still, further increase of 2,4-D level to 2μM/l, highest biomass production was observed (3550±25 mg and 235±06 mg) as seen in Fig.18a. Overall increase in biomass values recorded were 10 fold in terms of fresh and dry weights when compared with the inoculum. Increase in 2,4-D level to 4μM/l declined the growth of callus tissues (Fig.17).

Leaflet callus (300±20 mg,fr.wt. and 20±02 mg, dry wt.) cultured on medium without 2,4-D resulted in an increase in the biomass values, reaching to 650±03 mg and 45±02 mg in terms of fresh and dry weights at the end of four weeks culture period. Incorporation of 2,4-D at 0.5μM/l brought an increase in the biomass to 2000±20 mg and 135±02 mg in terms of fresh and dry weights respectively. This increase in callus biomass

Fig.17 Growth of stem / leaflet callus tissues on

MS + Sucrose (2%) + Kn (1.0 μM /l) + 2, 4-D (0, 1, 2 and 4 μM /l)

Culture period : Four weeks

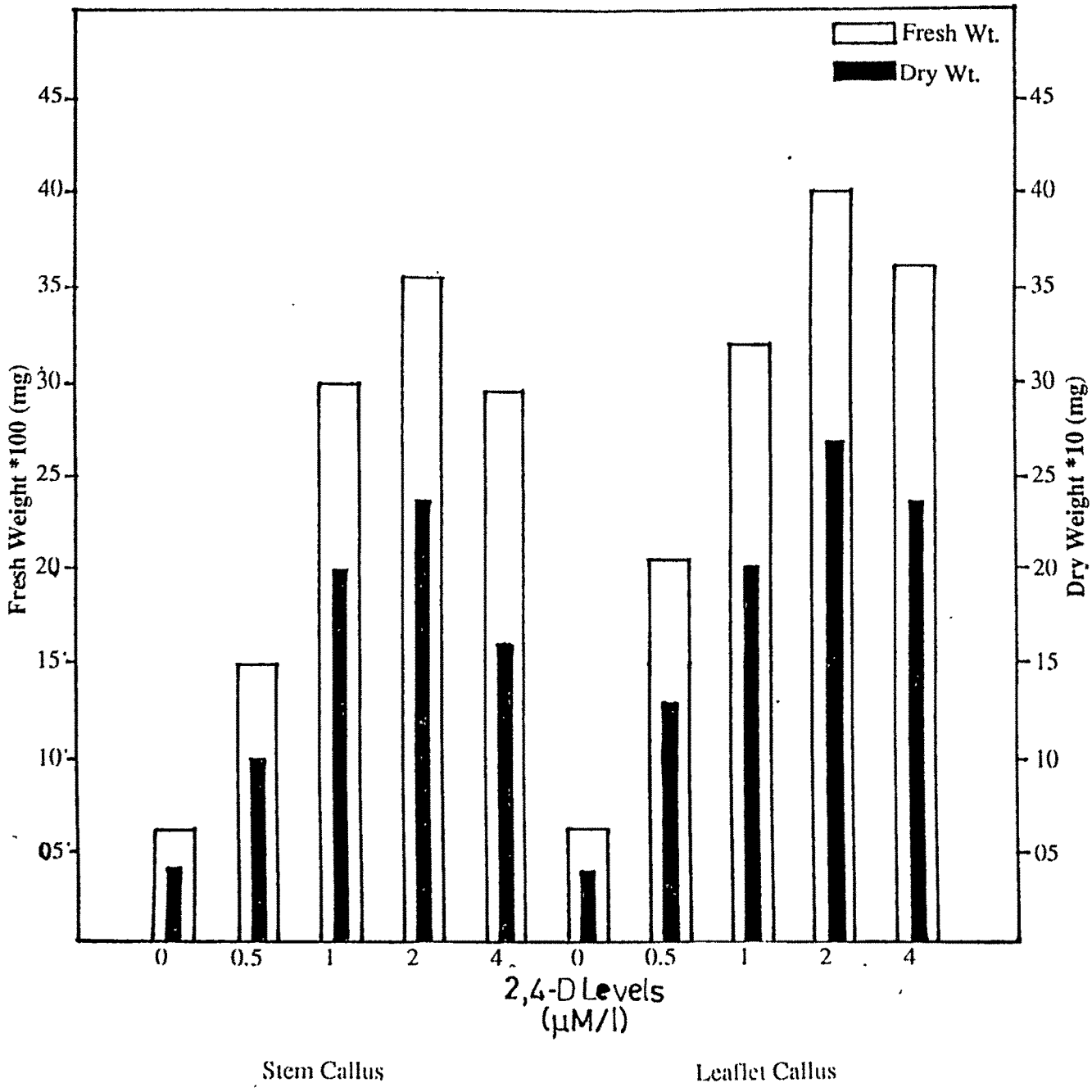

Fresh Wt.
Dry Wt.
Fresh Weight *100 (mg)
Dry Weight *10 (mg)
45
40
35
30
25
20
15
10
05
0
0.5
1
2
4
2,4-D Levels
(µM/l)
Stem Callus
Leaflet Callus

Fig.17

Fig.18 Biomass Production

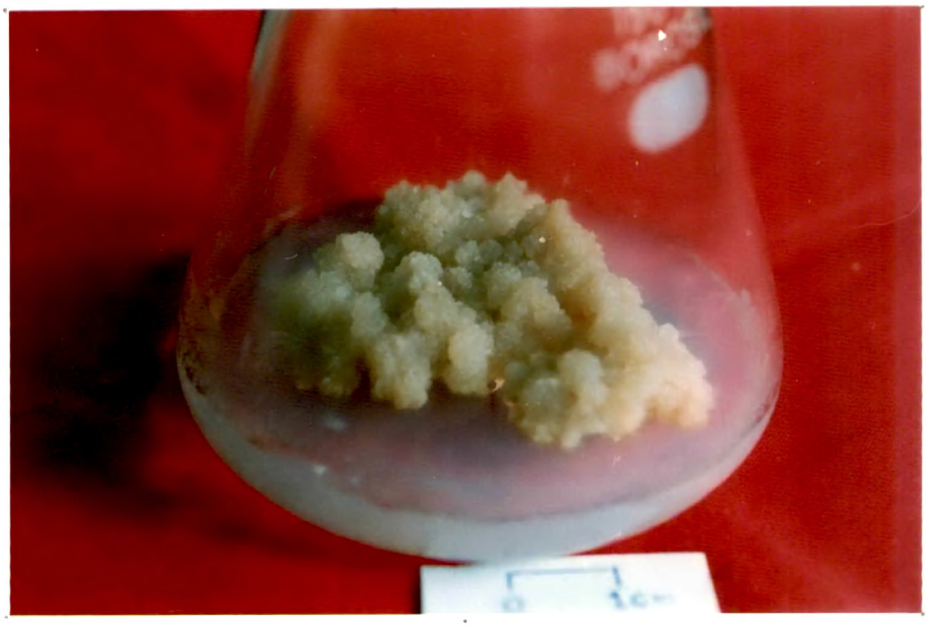
a) Stem callus

b) Leaflet callus

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

Culture period : Four weeks

a



b

Fig.18

continued with an increase in 2,4-D level, highest being at 2μM/l (fr.wt., 4000±30 mg and dry wt., 265±02 mg) as seen in Fig.18b. Further, increase in 2,4-D level to 4μM/l resulted in a decline in biomass production (fr.wt., 3600±42 mg and dry wt., 235±03 mg).

Thus, it was evident that 2,4-D at 2μM/l was the optimal level for maximum biomass production of both the callus tissues. Hence, MS medium (30 ml) containing sucrose (2%) supplemented with Kn (1μM/l) in combination with 2,4-D (2μM/l) was designated as 'Standard Medium' for callus cultures. Hence, in further experiments this medium was used.

Experiment 10 : Quercetin profile of callus cultures

The biosynthetic ability of the stem and leaflet callus tissues for quercetin synthesis/accumulation during eight weeks culture period was examined in the present experiment.

About 300±20 mg of healthy callus tissues of stem/ leaflet were inoculated on standard medium (30ml). The culture

flasks were incubated at 25±2°C in 16h photoperiod (1000 lux) in white flourescent light.

Results in Fig.19 showed that the stem callus tissues in terms of fresh and dry weights registered 500±20 mg and 35±05 mg biomass values at the end of first week. In the second and third week their values reached to 1200±40 mg and 80±03 mg; 3200±25 mg and 210±06 mg in terms of fresh and dry weights respectively. Further, with the passage of time, at the end of four and eight weeks, the fresh and dry weight values were 3500±10 mg & 230±01 mg; 3600±20 mg and 235±03 mg respectively.

During eight weeks period, chemical analysis of stem callus culture indicated that in first three weeks there was no indication for the presence of quercetin. However, its presence was detected at the end of four weeks. Finally, at the end of eight weeks, 0.007% of quercetin was accumulated in the stem callus.

Fig.19 Quercetin profile of stem and leaflet callus tissues during eight weeks period

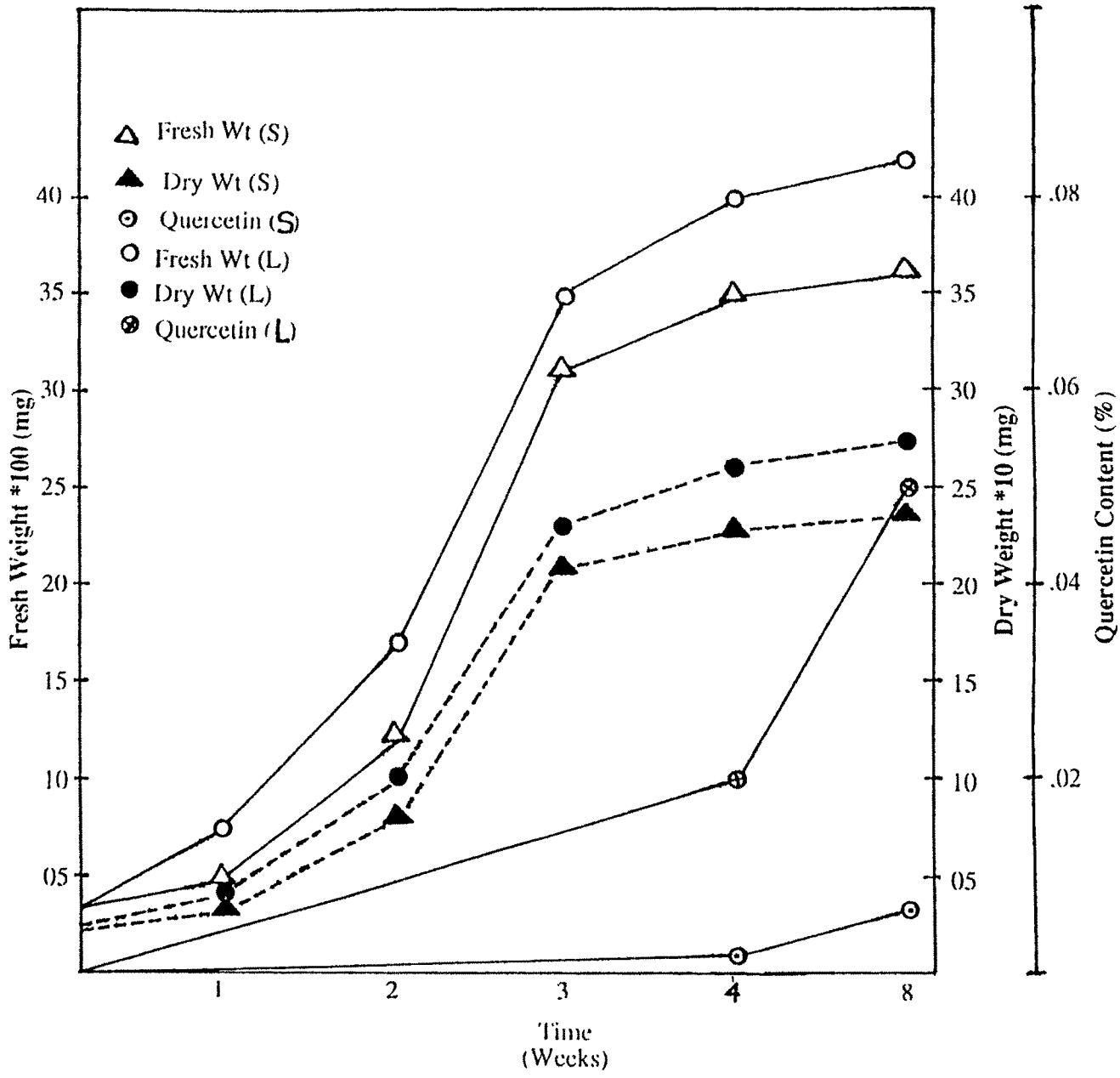

Fresh Wt (S)
Dry Wt (S)
Quercetin (S)
Fresh Wt (L)
Dry Wt (L)
Quercetin (L)
Fresh Weight *100 (mg)
Dry Weight *10 (mg)
Quercetin Content (%)
40
35
30
25
20
15
10
05
.08
.06
.04
.02
1
2
3
4
8
Time
(Weeks)

Fig. 19

In the leaflet callus, the biomass values in terms of fresh and dry weights at the end of first week were 750±10 mg and 42±02 mg. Subsequently, at the end of second week, 1750±30 mg & 110±02 mg biomass values in terms of fresh and dry weights were recorded. With the passage of time, biomass values increased two fold in the third week (Fig.19) and attained maximum values in the fourth week (fr.wt.,4000±25 mg; dry wt., 262±03 mg). Further, at the end of eight weeks, fresh and dry weights reached to 4200±10 mg and 270±02 mg respectively.

Chemical analysis of leaflet callus cultures for finding out the amount of quercetin synthesised at weekly intervals for a period of eight weeks was conducted. It was observed (Fig.19) that at the end of first two weeks, callus failed to synthesise/ accumulate quercetin while its presence was detected at the end of third week. Subsequently, at the end of four and eight weeks 0.02% and 0.05% quercetin were quantified.

The experimental result proved that stem and leaflet callus cultures exhibited the capacity for synthesis/ accumulation of quercetin. When comparison of the quercetin content accumulated by the stem and leaflet callus tissues was made, it was observed that leaflet callus tissues possessed better biosynthetic ability for the synthesis of quercetin.

Experiment 11 : Morphogenic potential of callus tissues

In order to achieve shoot-bud regeneration from stem/ leaflet callus tissues, the present experiment was conducted.

Healthy callus tissues (1-2 cm in dia.) maintained for a week on MS basal media were transferred to media containing Kn/BAP (1,2,4 and 6µM/l) alone or in combination with auxins *viz.*, IAA/NAA/IBA (0.5, 1 and 2µM/l) to find their effects on exhibiting a morphogenic response.

Results recorded (Table VIII) indicate that stem callus in presence of Kn, at all the levels tested, failed to

Table VIII : Morphogenic response of callus tissues

Medium : MS + Sucrose (3%) + Kn/BAP/ (BAP + IAA/NAA/IBA)

Inoculum : Callus tissues (1-2 cm in dia.)

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

Sr. No.	Growth Regulators (μM/1)	Morphogenic Response		% Response	
		Stem callus	Leaflet callus	Stem callus	Leaflet callus
	Kn				
1	(1-6)	-	-	-	-
	BAP				
2	1.0	-	-	-	-
3	2.0	-	-	-	-
4	4.0	green	green	70	90
5	6.0	-	-	-	-
	BAP + IAA				
6	4.0 0.5	-	green & NC	-	40
7	4.0 1.0	-	-	-	-
8	4.0 2.0	-	-	-	-
	BAP + NAA				
9	4.0 0.5	green & NC	green & NC	40	90
10	4.0 1.0	-	NC	-	60
11	4.0 2.0	-	-	-	-
	BAP + IBA				
12	4.0 0.5	-	-	-	-
13	4.0 1.0	-	-	-	-
14	4.0 2.0	-	-	-	-

Mean of six replicates
- No response
NC Nodular callus

exhibit any morphogenic response at the end of four weeks. Replacing Kn with BAP (1 and 2 µM/l), callus grew without any sign of morphogenesis while at its 4µM/l callus turned green. Increase in BAP level to 6µM/l, callus formation was promoted without any morphogenic response. Thus, out of the BAP levels tested, 4µM/l proved to be the most responsive treatment for chlorophyll synthesis in callus tissues.

Leaflet callus when cultured on media supplemented with Kn at all the levels or BAP (1/2 µM/l) failed to show any response in four weeks. Increase in BAP level to 4 µM/l, callus turned green and compact in 90% of the cultures. Further increase in BAP level to 6µM/l, no response was elicited by the callus tissue.

Stem callus tissue when subcultured on media supplemented with BAP (4µM/l) and IAA (0.5, 1 and 2µM/l) induced profuse callus growth without eliciting any morphogenic response. Keeping BAP level constant in the medium and supplementing it with NAA (0.5µM/l), green and compact callus

tissue turned nodular. Increase in NAA level to 1 and 2μM/l, callus tissue remained without any morphogenic response.

Maintaining BAP level constant (4μM/l), in combination with IBA (0.5μM/l), callus tissue failed to grow without any response while at its 1 and 2μM/l, callus grew at a rapid rate.

Leaflet callus grown on BAP (4μM/l) with IAA (0.5μM/l) induced nodules in 40% of the cultures while at 1 and 2μM/l of IAA, no morphogenic response was observed. Keeping BAP level constant (4μM/l) when NAA was supplemented at 0.5μM/l, callus turned nodular in 90% of the cultures (Fig.20) while at its 1μM/l level 60% cultures turned nodular. Increasing NAA level to 2μM/l, leaflet callus failed to show any morphogenic response.

Replacing NAA with IBA at all the levels tested (0.5,1 and 2μM/l) in combination with BAP (4μM/l) there was no response shown by callus tissues (Table VIII).

Fig.20 Leaflet callus - nodular and green

- After four weeks

Fig.21 Photomicrograph of nodule showing embryonic cells (10X x 25X)

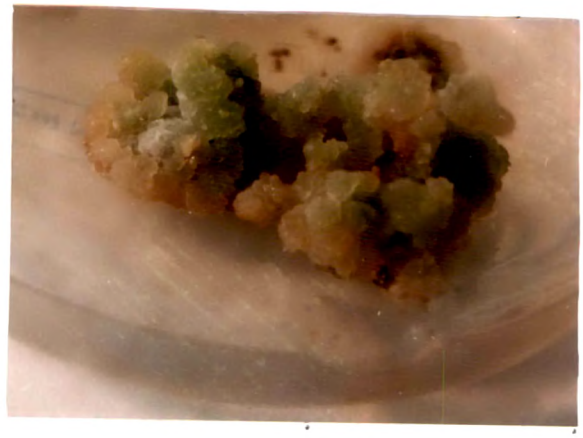


Fig. 20

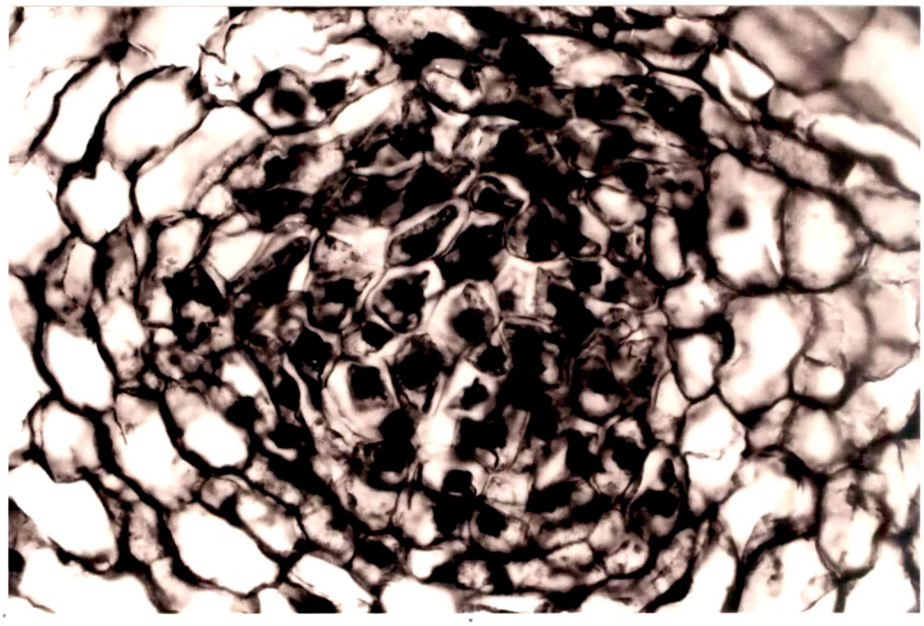


Fig. 21

Microscopic observation of nodular callus revealed that it consisted of thin walled, concentrically arranged embryonic cells with prominent nuclei (Fig.21). These nodules represented the meristematic growth centres having the capacity for organogenesis.

In order to differentiate this callus into shoot-buds, it was subcultured on media supplemented with BAP at 0.5,1 and 2µM/l. Culture flasks were incubated in the usual manner. It was interesting to observe (Table IX) that media without BAP failed to differentiate the nodular callus into shoot-buds. Supplementing the medium with BAP at 0.5µM/l induced two shoot-buds while at 1µM/l, four shoot-buds differentiated as seen in Fig.22. Further, increase in BAP level to 2µM/l resulted in a decline in the number of shoot-bud differentiation (Table IX).

Hence, BAP (1µM/l) proved superior for shoot-bud differentation from nodular callus. From the results of this experiment, it is concluded that medium supplemented with

TABLE IX : Differentiation of shoot-buds from leaflet callus

Medium : MS + Sucrose (3%) + BAP

Inoculum : Leaflet callus tissues (1-2 cm in dia.)

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

Sr. No.	BAP level (μM/1)	Number of shoot-buds differentiated from nodular callus	% response
1	0.0	0	-
2	0.5	2	70
3	1.0	4	90
4	2.0	1	65

Mean of six replicates
- No response

Fig.22 Differentiation of shoot-buds (S.B.) from leaflet callus

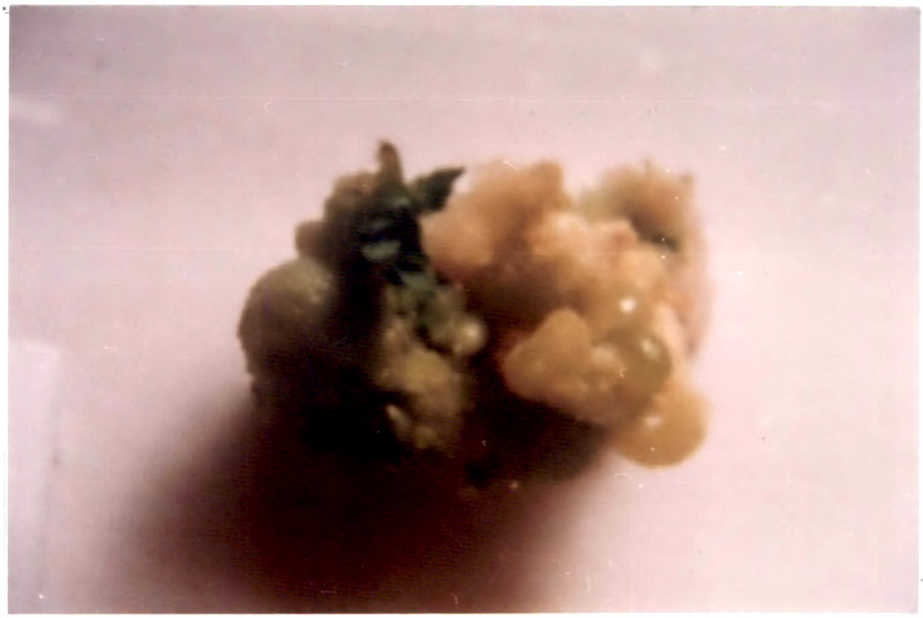


Fig. 22

BAP (4μM/l) and NAA (0.5 μM/l) was suitable for eliciting best morphogenic response. This callus when subcultured on medium supplemented with BAP (1μM/l) facilitated shoot-bud differentiation.

Experiment 12 : Elongation of shoot-buds

Shoot-buds produced either from axillary buds or from callus cultures need to be elongated to suitable length to develop adequate number of foliage. Individual bud on isolation was cultured MS medium containing sucrose (3%) and GA_3 at various levels (0.5,1,2 and 4μM/l) for its elongation. Six replicates per treatment were maintained in the culture room at 25±2°C in 16h photoperiod (1000 lux).

It was noticed that in response to GA_3 (0.5μM/l) the shoot-buds elongated upto length of 1.6±0.24cm in 50% of the cultures as seen in the results of Table X after four weeks of culture period. At the same time, shoot-buds cultured on 1 and 2μM/l of GA_3 elongated to 4.0±0.25 cm and 8.0±0.56cm in length respectively with 90% response. Further increase in

TABLE X : Effect of Gibberellic acid on shoot elongation

Medium : MS + Sucrose (3%) + GA_3

Inoculum : Isolated shoot -bud (1-1.5 cm)

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

Sr. No.	GA_3 levels (μ M/1)	Length of shoot (cm)	% response
1	0.5	1.6 ± 0.24	50
2	1.0	4.0 ± 0.25	90
3	2.0	8.0 ± 0.56	90
4	4.0	1.8 ± 0.20	80

Mean of six replicates with standard deviation

GA_3 level to 4µM/l in the culture medium could not enhance the length of the cultured shoot.

Results of this experiment indicated that 2µM/l of GA_3 treatment produced shoots with maximum length.

In order to produce well developed foliage on these elongated shoots, $AdSO_4$ was incorporated at various levels *viz.*,1,2 and 4µM/l in the culture media. Shoots cultured on 1µM/l of $AdSO_4$ supplemented media developed leaves with small sized lamina(Fig.23a). Moreover, these leaves were pale green in colour. In response to 2µM/l of $AdSO_4$, the shoots produced healthy well developed foliage (Fig.23b), besides the foliage was dark green. At 4µM/l of $AdSO_4$ in the culture medium, no beneficial effect was found on the cultured shoots either on the lamina size of leaf or its chlorophyll content (Fig.23c). Hence, 2µM/l of $AdSO_4$ was found suitable concentration for the production of well developed foliage on the elongated shoots.

Fig.23 Shoots cultured on media containing $AdSO_4$

a) 1μM/l

b) 2μM/l

c) 4μM/l

showing variation in foliage size

a

b

c

Fig. 23

Experiment 13 : Plant formation

In vitro developed shoots produced either by axillary bud sprouting or by callus cultures were to be developed into plants. Hence, these shoots were transferred to half strength MS media containing sucrose (1%) and supplemented with various auxins *viz.*, IAA/NAA/IBA (0.5,1 and 2µM/l). Cultures were incubated for four weeks at 25±2°C in 16h photoperiod (1000 lux).

Results recorded in Table XI indicate that in response to IAA at 0.5µM/l, weak hairy root was produced which callused. Increase in IAA level to 1/2µM/l resulted in friable callus formation at the base of shoot without root initiation.

Well developed excised shoots when cultured on IBA at 0.5µM/l induced a root initial from 6th day onwards. Subsequently, a healthy tap root system with secondary roots was formed in 25-30 days (Fig.24). Increasing IBA level to 1µM/l resulted in induction of a root in 60% of the cultures tested. This root failed to elongate further as it started

TABLE XI : Plant formation

Medium : Half strength MS medium + Sucrose (1%) + IAA/IBA/NAA

Inoculum : Single shoot (>2 cm)

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

Sr. No.	Auxin levels (μM/1)	Induction of Roots/ Callus	% response
	IAA		
1	0.5	Callus + Root	50
2	1.0	Callus	-
3	2.0	Callus	-
	IBA		
4	0.5	Tap Root	80
5	1.0	Root + Callus	60
6	2.0	Callus	-
	NAA		
7	0.5	Root + Callus	70
8	1.0	Root + Callus	50
9	2.0	Callus	-

Mean of six replicates

Fig.24 Development of a tap root system from well developed shoot in MS/2 + Sucrose (1%) + IBA (0.5µM/l)

Fig. 24

callusing after slight growth. Further, increase in IBA level to 2μM/l resulted in profuse callus formation.

Replacing IBA with NAA at 0.5μM/l, thin and fragile root was induced from the base of shoot while at 1μM/l of it, thick and stunted 1-3 roots with callus were produced. Increasing NAA level to 2μM/l, cultured shoots failed to exhibit any morphogenic response instead profuse callus was produced at the base of the shoot.

Hence, out of the auxins tested, IBA at 0.5μM/l proved suitable for the development of a healthy tap root system, thus establishing a well developed healthy plant.

Experiment 14 : Acclimatization and transfer of plants to pots

In vitro developed plants were washed with distilled water and subsequently transferred to test tubes with sterile distilled water for 8-10 days. This resulted in hardening of the root system. These hardened plants were transferred to pots containing sterilised vermiculite. Subsequently, they were covered with glass beaker (Fig.25) for first 4-5 days with intermittent exposure to air for few hours every day. Gradual removal of beaker provided the plants with sufficient period for adjustment to environmental conditions. Further, plants were transferred to pots containing coarse sand and soil (1:3 v/v) where they were watered every second day. These plants once established in pots were gradually transferred to field where they recorded 90% survival at the end of one year period.

Fig.25 Transfer of *in vitro* developed plantlet to vermiculite covered with glass beaker

Fig.26 Root tip squash showing diploid (2n=20) chromosomes at the metaphase plate

Fig. 25

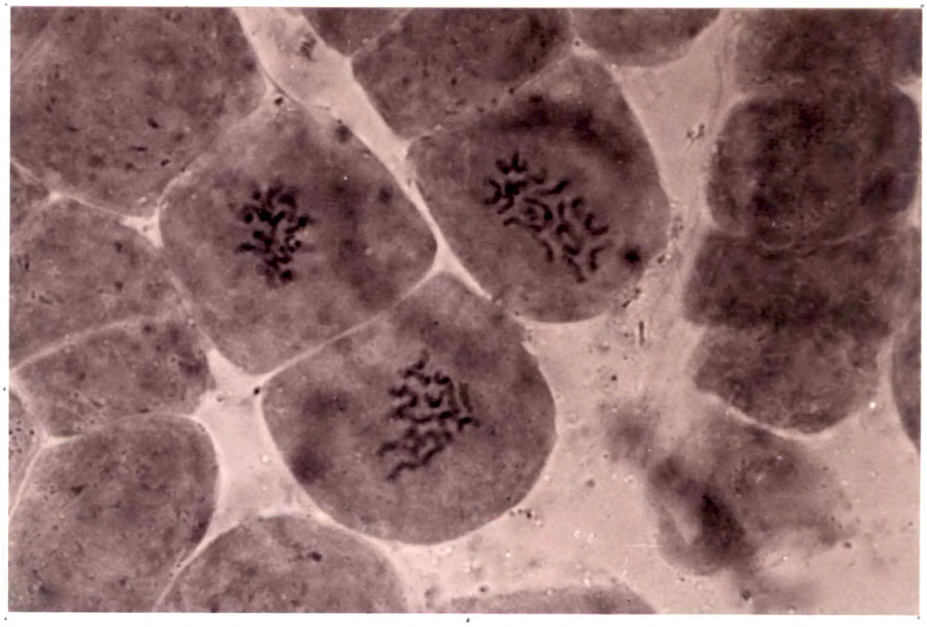


Fig. 26

Experiment 15 : Determination of ploidy of the regenerated plants

In order to determine the ploidy of the regenerated plants, their root-tip squash preparations were made as described in Materials and Methods, Chapter II.

Cells with metaphase plate were observed and their chromosome number was noted. All the cells revealed the presence of 2n=20 chromosomes (Fig.26). Thus, proving the diploid nature of the regenerated plants.

ALBIZZIA LEBBECK BENTH.

Albizzia lebbeck Benth. is a deciduous, timber yielding ornamental tree (Fig.27a,b). Extracts of its stem-bark and leaves are used in the preparation of Ayurvedic drugs for the treatment of respiratory diseases and eczema. The medicinal importance of this plant is due to the chemical compounds synthesised during the metabolic processess as a result of growth and development. These compounds are flavonoids.

SECTION A : *IN VIVO* STUDIES

In this section, chemical analysis of the samples of stem-bark and leaves of mature *A.lebbeck* trees growing in and around Baroda was conducted for the presence of a medicinally important flavonoid *viz.*, quercetin. Based on the results, an 'elite' superior tree of *A.lebbeck* was to be selected. Further, explants from it were used for propagation of this tree.

Experiment 16 : Selection of 'elite' *A.lebbeck* plant

Healthy stem-bark and leaf samples from *A.lebbeck* plants growing in (i) Subhanpura (ii) Sama (iii) I.P.C.L

Fig.27 a) A *Albizzia lebbeck* Benth. tree growing in Subhanpura

b) A flowering twig

a

b

Fig. 27

township (iv) Botanical garden of M.S. University of Baroda were collected. These samples were washed under tap water, air dried, powdered and kept in labelled bottles.

The powder samples of stem-bark and leaves (40gm) were soxhlet extracted using 200ml of methanol (80%) for 48h employing the procedures described by Harborne (1962) as given in Chapter II, Materials and Methods. Methanol, the solvent used was then distilled off and the residue was redissolved in water and filtered. The filtrate was hydrolysed with HCl (7%), concentrated by heating it on a waterbath at 100°C. The residue was dissolved in ethylacetate and the two fractions, one water soluble and other ethy aceate soluble were isolated by using separating funnel. Subsequently, the ethyl acetate soluble fraction was concentrated by evaporating the solvent. The residue was loaded on the paper chromatogram in the form of a band for the separation of flavonoids using acetic acid (30%) as the solvent system. Similarly, authentic sample of quercetin was loaded on a chromatogram. Samples under investigation developed yellow coloured band (Fig.28) in

Fig.28 Paper chomatogram of stem-bark sample showing the presence of quercetin (Y)

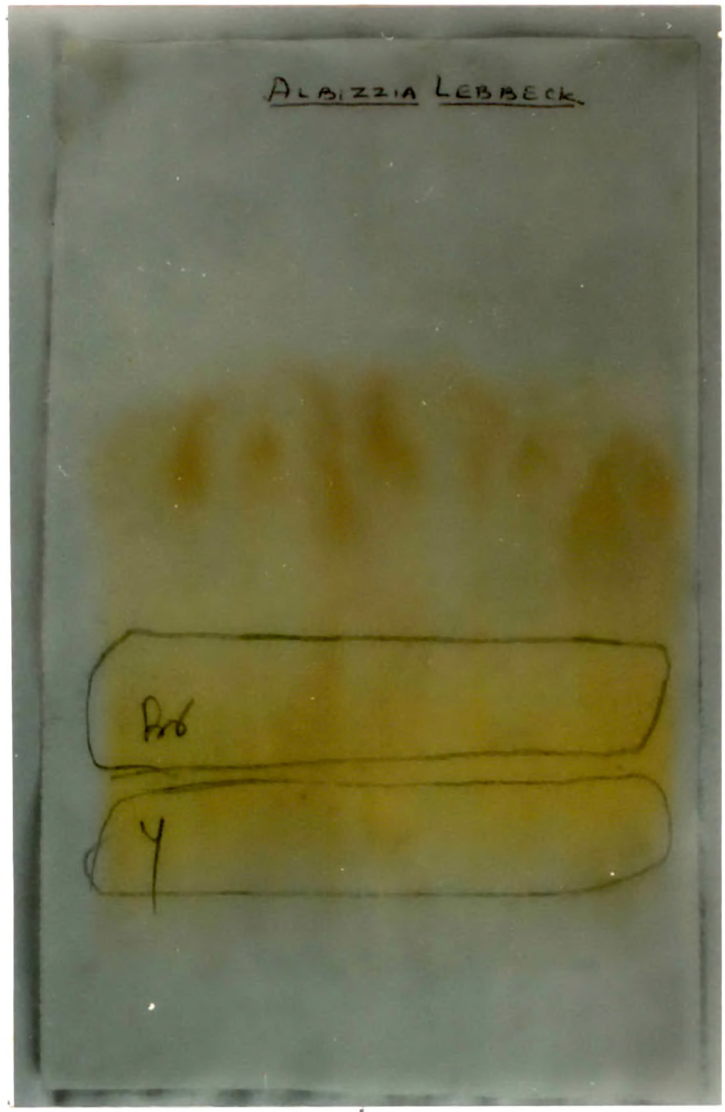


Fig. 28

sodium carbonate solution. Similar yellow coloured band was observed in the authentic sample of quercetin indicating the presence of quercetin in the sample. All other samples when screened indicated the presence of quercetin in them.

Spectrophotometric analysis of the compound isolated from the leaf samples developed superimposable spectra with the authentic sample of quercetin (Fig.29). This confirmed the presence of quercetin in leaf sample.

Quercetin isolated from each of the sample was quantified following the procedures of Block and Durrum (1958) as given in Chapter II, Materials and Methods. However, presence of quercetin was detected in all the four stem-bark samples but their quantities being very low, they could not be quantified.

Leaf sample of *A.lebbeck*, Subhanpura area growing plant synthesized/accumulated 1.02% quercetin while the leaves of Botanical garden growing plant accumulated 0.85% of

Fig.29 U V absorption spectra of quercetin
1) Authentic sample
2) Leaf sample of *A.lebbeck*

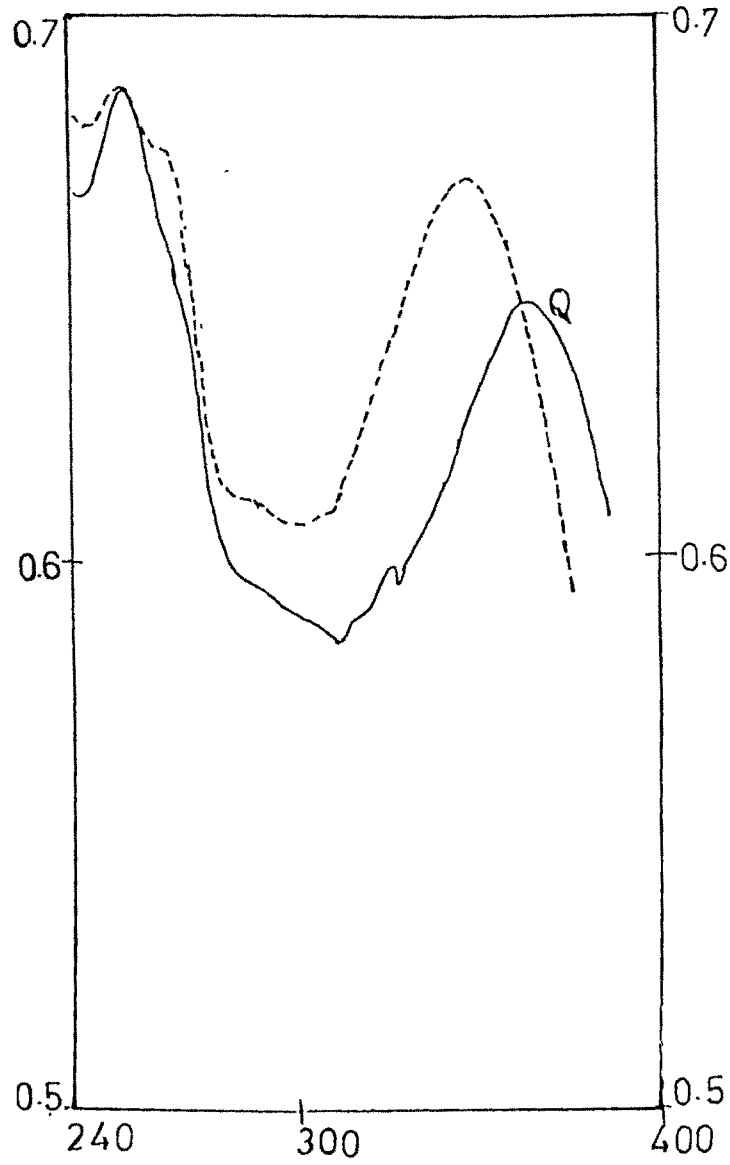


Fig. 29

quercetin when calculated on percentage dry weight basis. Leaf sample of Sama area growing plant showed the presence of 0.70% quercetin. Lowest quantity of quercetin, 0.62% was isolated from leaves of *A.lebbeck* plants growing in I.P.C.L. township (Table XII).

Results of this experiment proved that stem-bark and leaves of *A. lebbeck* synthesized/accumulated quercetin. Quantitative estimation revealed that very low quantities of quercetin were accumulated in four stem-bark samples. Leaves of the plants under investigation accumulated quercetin in appreciable amounts, highest quantity was detected in Subhanpura area growing plant. Hence, *A.lebbeck* plant growing in Subhanpura was selected as an 'elite' superior plant.

SECTION B : *IN VITRO* STUDIES

This section deals with the experimental work conducted for the standardisation of a protocol for rapid multiplication and propagation of 'elite' *A.lebbeck* plants

TABLE XII : Quercetin profile in leaf samples of *Albizzia lebbeck* Benth.

Sr No.	*A. lebbeck* growing in various localities	Quercetin content (%)
1	Subhanpura	1.02
2	M S.University Botanical garden	0.85
3	Sama	0.70
4	I.P.C.L Township	0.62

% dry weight basis

through its axillary bud sprouting as well as through induction of adventitious buds and callus cultures.

I. AXILLARY BUD SPROUTING

Experiment 17 : Effect of cytokinin/s (Kn/BAP) alone or in combination on axillary bud sprouting

Axillary buds from 'elite' *A.lebbeck* were cultured on media supplemented with Kn and BAP alone or in combination, in order to induce their sprouting into shoot-buds. The optimal level of cytokinin/s required for maximum number of healthy shoot-bud induction from an axillary bud has been determined. These buds were employed for rapid multiplication and propagation of this species.

a) Effect of Kn

Healthy nodal segments with an axillary bud, second, third, fourth and fifth from the apex of a branch in serial order of development from an 'elite' *A.lebbeck* plant were collected in a flask containing water. They were washed under tap water

and surface sterilised according to the procedures described in Chapter II, Materials and Methods. Each axillary bud was inoculated in Erlenmeyer flask (150ml) containing MS (30ml) basal medium with sucrose (3%) and supplemented with Kn (0,1,2,4 and 6µM/l) pH-5.8 individually by inserting it in the culture medium. Culture flasks were incubated at 25±2°C in a culture room in 16h photoperiod (1000 lux).

Results recorded in Table XIII indicate that each bud cultured on basal medium without Kn-turned black within one week culture period while buds grown on 1µM/l of Kn containing medium were swollen. Increasing the level of Kn to 2µM/l, the original bud grew into a shoot in 70% of the cultures (Fig. 30a). The third bud in serial order of development produced healthy vigorous shoot while the shoots produced from second, fourth and fifth buds were smaller in size Hence, the third axillary bud was used as an explant for multiplication. Further, increase in Kn level to 4/6µM/l resulted in growth of the original bud into a shoot-bud with callus at the base.

TABLE XIII : Effect of cytokinin/s on axillary bud proliferation

Medium : MS + Sucrose (3%) + Kn/BAP

Inoculum : A nodal segment with an axillary bud

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

Sr No.	Cytokinin/s level (μM/l)		No. of shoot-buds induced/explant	% response
	Kn	BAP		
1	0	-	-	-
2	1	-	-	-
3	2	-	1	70
4	4	-	1 + C	60
5	6	-	1 + C	60
6	-	1	1	50
7	-	2	2±0.20+C	60
8	-	4	3±0.85+C	80
9	-	6	1 + C	70
10	2	2	3 ±0.56	70
11	2	4	5±0 10	80
12	2	6	2±0.25	60
13	4	2	2±0.52	50
14	4	4	3±0 24	60
15	4	6	3±0 41	50
16	6	2	2±0.25	60
17	6	4	2±0.05+C	60
18	6	6	1 + C	50

Mean of six replicates with standard deviation
C - Callus

Fig.30 Axillary bud (third) cultured on MS+ Sucrose (3 %)
(a) + Kn (2μM/l)

(b) + BAP (4μM/l)

(c) + Kn (2μM/l) + BAP (4μM/l)

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

a

b

c

Fig. 30

It was evident that the third axillary bud developed into a single healthy shoot in presence of 2µM/l of Kn. At other levels of Kn tested axillary bud either failed to grow or grew into dwarf shoot-bud with callus. Thus, Kn alone proved unsuitable for sprouting of the axillary bud.

b) Effect of BAP

MS medium (30ml) containing sucrose (3%) was supplemented with BAP (0,1,2,4 and 6µM/l). Surface sterilized third axillary buds from apex were inoculated in 150ml Erlenmeyer flasks containing above media and they were incubated as per the procedures described in Chapter II, Materials and Methods.

Results recorded at the end of four weeks (Table XIII) showed that axillary bud grew into stunted shoot-bud in presence of 1µM/l of BAP. Increase in BAP level to 2µM/l, resulted in sprouting of the axillary bud into 2±0.20 shoot-buds with compact callus from the cut ends. At 4µM/l of BAP, the number of buds sprouted from the third axillary bud were 3±0.85

in 80% cultures (Fig.30 b). These buds were healthy with callus. Further, increase in BAP level to 6μM/l, bud sprouting was arrested but it grew into a single thick and fleshy shoot.

Out of the BAP levels tested, 4μM/l was found better as it induced sprouting of axillary bud. It could be concluded that a single cytokinin failed to induce sprouting of an axillary bud into highest number of shoot-buds. Hence, a suitable combination of Kn with BAP was to be determined which would increase the number of sprouted shoot-buds.

c) Effect of Kn in combination with BAP

In order to increase the number of sprouted buds from a single axillary bud, the effect of Kn in combination with BAP at various levels was tested.

Erlenmeyer flasks (150 ml) containing MS media (30 ml) with sucrose (3%) and Kn (2,4 and 6μM/l) in combination with BAP (2,4 and 6 μM/l) were incoulated with nodal segments consisting of third axillary buds after subjecting them to surface

sterilization procedures. Culture flasks were incubated as usual.

Results recorded (Table XIII) indicate that Kn and BAP each at 2μM/l induced swelling of the axillary bud in one week which subsequently sprouted into 3 ± 0.56 shoot-buds in four weeks (70%). Keeping Kn level constant and increasing BAP level to 4μM/l resulted in sprouting of axillary buds into 5±0.10 robust, separable buds in 80% of the cultures. Each of these buds reached to a height of 4-5 cm with 1-2 nodes (Fig.30c). These healthy sprouted shoot-buds when subcultured individually on fresh medium of the same composition produced a crop of 4-5 vigorous shoots. Growth of these newly sprouted buds was faster. Further increase in BAP level to 6μM/l with Kn (2μM/l) reduced the rate of sprouting of the axillary bud into 2±0.25 buds (60%).

Increasing Kn level to 4μM/l in combination with BAP (2μM/l) there was sprouting of the bud into 2±0.52 shoot-buds in 50% of the cultures. Keeping Kn level constant and

increasing BAP level to 4 and 6µM/l resulted in sprouting of the axillary bud into 3±0.24 and 3±0.41 shoot-buds respectively in 60% and 50% of the cultures.

Further increase in Kn level to 6µM/l with BAP (2µM/l) there was a decline in the number of shoot-buds sprouted to 2±0.25 per explant while at 4µM/l of BAP axillary buds sprouted into 2±0.05 buds with callus at the base. Thus, Kn at 4 and 6µM/l in combination with BAP at all the levels tested failed to enhance the shoot-bud number.

Hence, Kn (2µM/l) with BAP (4µM/l) produced synergistic effect as a result of which the axillary bud sprouted into maximum number of shoot-buds. This medium was designated as 'Multiplication Medium' for this species and was used in further experiments. In order to maintain the vitality of the shoots produced, their rate of multiplication was kept under control.

Experiment 18 : Effect of auxin (IAA/NAA) on axillary

bud proliferation

In order to find out the effect of addition of auxin/s *viz.*, indole-3-acetic acid (IAA) or ∝-napthalene acetic acid (NAA) to the multiplication media for the improvement in the rate of axillary bud sprouting, this experiment was conducted.

Surface sterilized nodal segments with third axillary buds were inoculated on MS multiplication media (control) supplemented with IAA/NAA at 0.5, 1 and 2µM/l in culture flasks and incubated maintaining similar experimental conditions.

Results recorded after four weeks (Table XIV) showed that the third axillary bud sprouted into 5±0.10 buds in 100% cultures in control. When this medium was supplemented with IAA (0.5µM/l), the axillary bud sprouted into 3.5±0.30 buds thereby reducing the number of fresh buds sprouted. In addition to it callus was induced from the cut ends of the explant. Increasing IAA level to 1µM/l, the number of buds sprouted was 4±0.51 in 80% of the cultures with profuse callus at the

TABLE XIV : Effect of auxins on axillary bud proliferation

Medium : Multiplication medium + IAA/NAA

Inoculum : An axillary bud

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

Sr. No.	Auxin level (µM/l)	No. of buds induced/explant	% response
I	Control-Kn (2 µM/l) + BAP (4µM/l)	5±0.10	100
	IAA		
2	0 5	3 5±0.30+C	70
3	1	4±0.51+ C	80
4	2	2±0.12+C	60
	NAA		
5	0 5	3±0.24 +C	70
6	1	2±0.36 +C	70
7	2	2±0.10+C	60

Mean of six replicates with standard deviation
C - Callus

proximal cut end of the explant (Fig.31). Axillary buds in response to IAA at 2μM/l in the medium resulted in reduction of the number of buds sprouted with large amount of callus around its base (Table XIV).

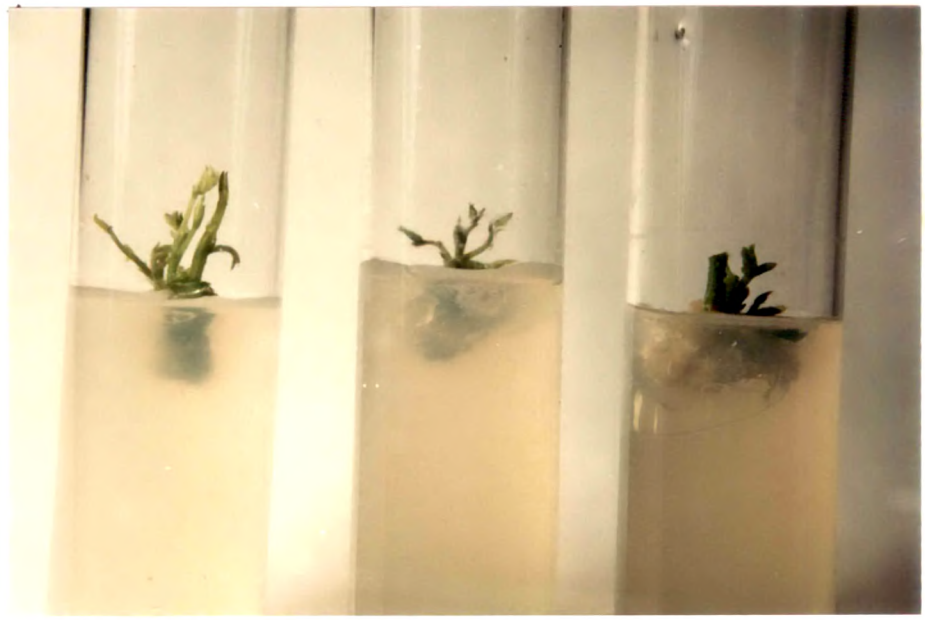
In NAA (0.5μM/l) incorporated medium, axillary bud sprouted into 3±0.24 buds along with basal callusing in 70% of the cultures. Increase in NAA level to 1/2μM/l resulted in a decline in the number of buds sprouted with profuse callus (Table XIV).

Thus, exogenous application of IAA/NAA at the concentrations tested, failed to enhance the number of buds sprouted. In addition to it, callus was induced from the base of the explant. Hence, these treatments proved unsuitable to achieve the objective of the present experiment.

Experiment 19 : Selection of suitable culture medium for axillary bud sprouting

Fig.31 Third axillary bud cultured on MS + Sucrose (3%)
(a) + Kn (2μM/l) + BAP (4μM/l) control
(b) Control + IAA (1μM/l)
(c) Control + NAA (1μM/l)

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



a b c

Fig. 31

In order to achieve highest number of shoot-bud proliferation from an axillary bud, a suitable formulation of nutrient medium is required to provide necessary nutrition to the bud for its sprouting. In the present experiment five known culture media *viz.*, Wood and Braun (WB, 1961), Murashige and Skoog(MS,1962); Gamborg's(B_5,1968); Schenk and Hilderbrandt (SH, 1972) and Lloyd and Mc Cown(LM, 1981) with their compositions as given in Table I (a-e), Materials and Methods were used to find out a suitable culture medium supporting highest number of shoot-buds from an axillary bud. These above mentioned media were supplemented with sucrose (3%), Kn (2µM/l) and BAP (4µM/l). Surface sterilized nodal segments with axillary buds were inoculated on above said media (30ml). Culture flasks were incubated in culture room at 25±2°C in 1000 lux light intensity for 16 h photoperiod.

Results recorded (Fig.32) indicate that 5±0.10 healthy, robust, fast growing, separable shoot-buds were produced from an axillary bud in MS medium in a period of four weeks. Axillary buds cultured on WB medium produced 3±0.12 stunted, swollen

Fig. 32 Axillary buds cultured on Sucrose (3%) + Kn (2μM/l) + BAP(4μM/l)+

a) Murashige and Skoog's medium
b) Wood and Braun medium
c) Gamborg's B_5 medium
d) Schenk and Hilderbrandt medium
e) Lloyd and Mc Cown medium

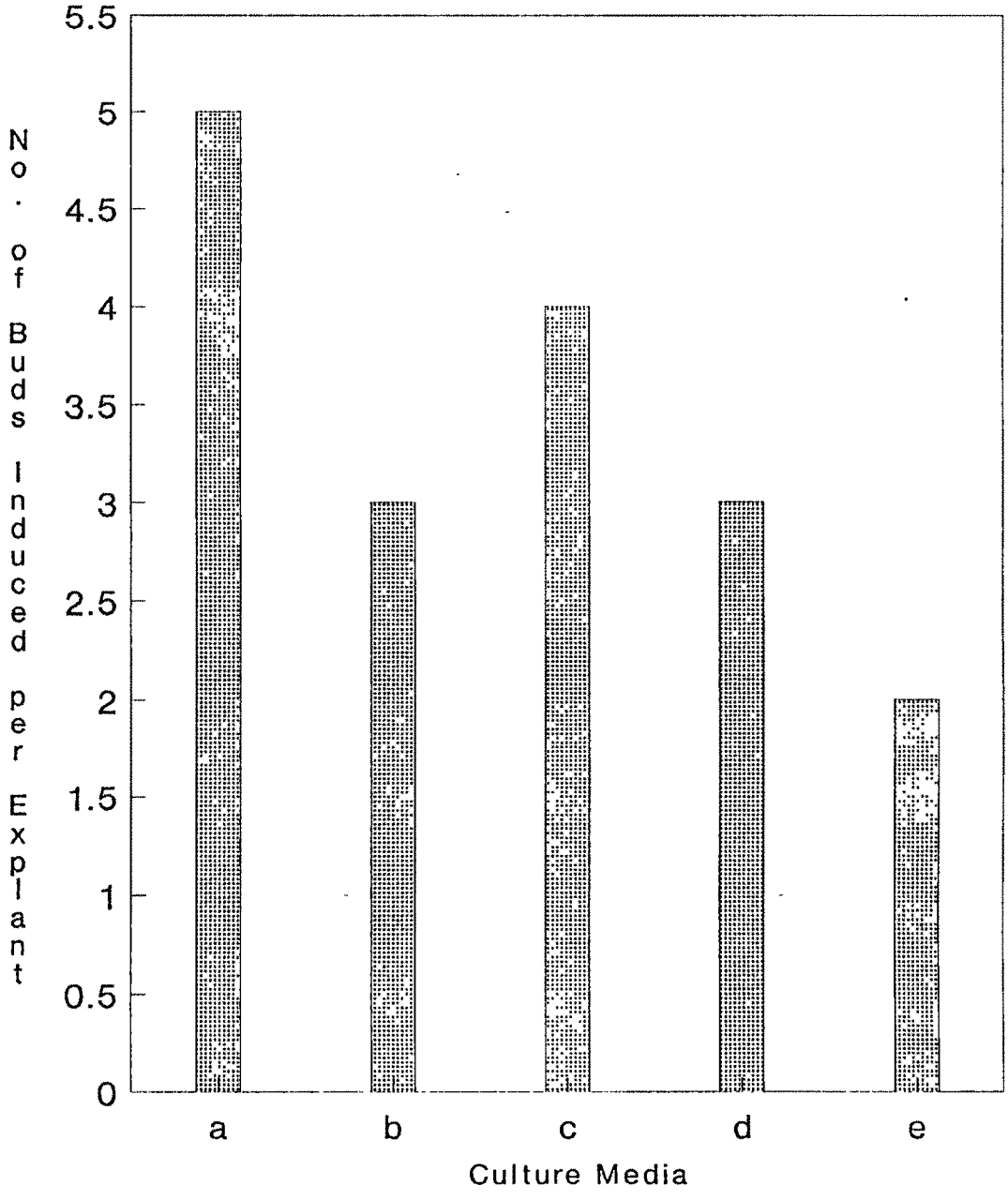

5.5
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
No. of Buds Induced per Explant
a
b
c
d
e
Culture Media

Fig. 32

buds with callus at the cut end of the nodal explant. Considerable leaf drop occured from the shoots cultured on WB medium. However, axillary buds cultured on B_5 medium sprouted into 4±0.05 shoot-buds per explant. This medium responded well intially but later produced extensive callus from the base of the explant. Axillary buds when cultured on SH medium sprouted into 3±0.15 shoot-buds per explant which grew at a slower rate. In LM medium, 2±0.30 thick and short shoot-buds sprouted from an axillary bud.

Thus, it was concluded that MS medium salts as present along with other supplements supported highest number of healthy shoot buds production. Hence, for further experiments MS medium was selected as a suitable culture medium.

Further, factoral experiments were conducted to find out the quantities of

(a) Inorganic nitrogen,

(b) Vitamins,

(c) Myo-inositol levels in MS medium required for optimal axillary bud sprouting.

a) Influence of inorganic nitrogen

In order to find out the effect of variations in the inorganic nitrogen levels of MS medium, they were adjusted as their absence, half dose, standard (840 mg/l) and double dose for induction of optimal number of shoot-buds from a single axillary bud. The ionic balance of the culture media was adjusted by using KCl.

Results recorded in Fig.33 indicate that axillary buds cultured in absence of inorganic nitrogen failed to grow and hence turned black. Inorganic nitrogen at its half dose produced two etiolated shoot-buds. However, in the standard dose of inorganic nitrogen five vigorously growing, easily separable shoot-buds were produced at the end of four weeks culture period. Doubling the level of inorganic nitrogen produced 3 thin shoot-buds per explant.

Fig. 33 Axillary buds cultured on MS medium in
a) Absence of inorganic nitrogen/ vitamins/ inositol
b) Half dose of " " " " " "
c) Standard " " " " " "
d) Double " " " " " "
containing Sucrose (3%) + Kn (2 µ M/l) + BAP (4µ M/l)

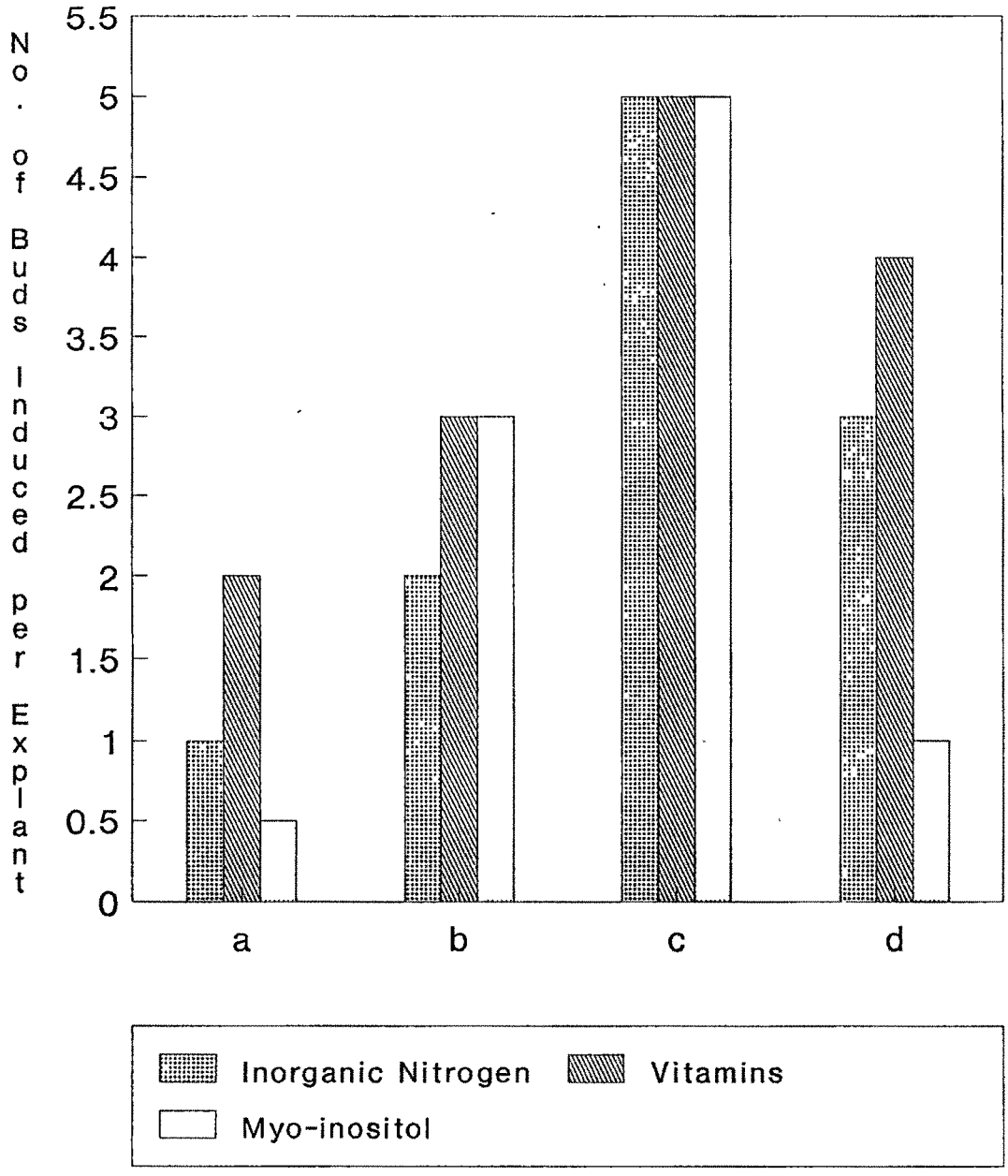

No. of Buds Induced per Explant
5.5
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
a
b
c
d
Inorganic Nitrogen
Vitamins
Myo-inositol
Media Constituents

Fig. 33

It was thus concluded that the level of inorganic nitrogen of MS medium was at its optimal level for sprouting of axillary bud of this species.

b) Influence of vitamins

MS media (30 ml) in absence /half dose/standard/ double dose of vitamins were evaluated for their influence on sprouting of optimal number of shoot-buds from an axillary bud. Cultural conditions were maintained the same.

Results depicted in Fig.33 indicate that in absence of vitamins axillary buds sprouted into 2±0.05 buds while in its half dose 3±0.20 buds per explant were produced. However, in standard dose of vitamins, 5±0.25 healthy shoot-buds per explant were produced. Doubling the vitamin level, single axillary bud produced 4±0.55 buds.

Hence, vitamins at the levels as present in MS medium were at their optimal level for achieving maximum number of shoot-bud proliferation per explant.

c) Influence of inositol

In this factoral experiment, MS media (30ml) in absence, half dose, standard and double dose of inositol were tested for their influence on axillary bud sprouting. Culture flasks were incubated following the procedures as described in Chapter II, Materials and Methods.

Results presented in Fig.33 indicate that in absence of inositol axillary buds failed to sprout while in its half strength, 3±0.25 buds sprouted along with callus at the base of explant. However, in standard dose of inositol, 5±0.50 buds per explant were produced. Further, doubling myo-inositol level arrested bud proliferation resulting in the growth of a single shoot bud with profuse callus from the cut end of the explant.

Thus, it was evident that for maximum proliferation of axillary bud, 100 mg/l of inositol as present in MS medium was at its optimal level.

Experiment 20 : Effect of various carbohydrates on axillary bud sprouting

Incorporation of carbon source to the culture medium serves as a source of energy required for the growth and development of the explant.

MS multiplication medium was supplemented with glucose/fructose/sucrose/maltose/mannitol/ starch at 3% and inoculated with surface sterilized third axillary bud following the culture procedures as described in Chapter II, Materials and Methods.

Results recorded in Table XV indicate that buds cultured on media supplemented with glucose produced 2±0.05 vitrified buds with large amount of callus at the base of explant in 70% of the cultures while in fructose supplemented media axillary bud produced 2±0.10 buds. However, in sucrose supplemented media, cultured axillary bud sprouted into healthy and separable 5±0.60 buds in 80% of the cultures tested (Fig.34). Axillary buds cultured on media supplemented with

TABLE XV : Effect of various carbohydrates on axillary bud proliferation

Medium : Multiplication medium + 3% (Glucose/Fructose/Sucrose/ Maltose/Mannitol/Starch)

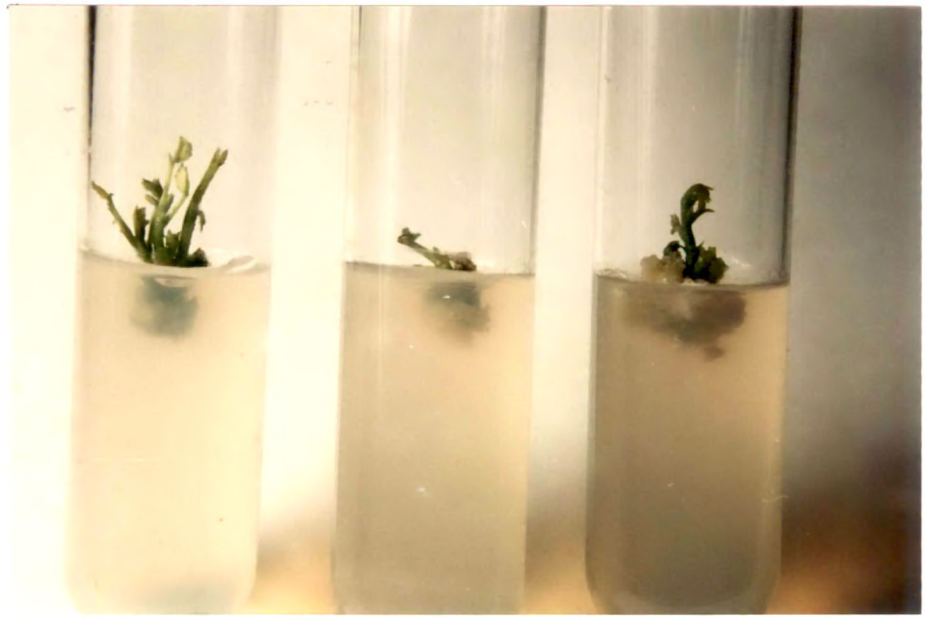
Inoculum : A nodal segment with an axillary bud

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

Sr. No.	Carbohydrates (3%)	Number of buds Induced per explant	% response
1	Glucose	2±0.05	70
2	Fructose	2 ± 0.10	60
3	Sucrose	5±0.60	80
4	Maltose	1	50
5	Mannitol	-	-
6	Starch	-	

Mean of six replicates with standard deviation

Fig. 34 Sprouting of third axillary bud cultured on multiplication media containing 3% of
a) Sucrose
b) Fructose
c) Glucose



a b c

Fig. 34

maltose induced a single slow growing shoot-bud while in mannitol and starch supplemented media bud turned brown and subsequently died.

Thus, sucrose proved to be the best carbohydrate source as in its presence maximum number of shoot-buds sprouted from single axillary bud. Further, the optimal sucrose concentration required for highest number of shoot-buds sprouting from a single axillary bud was determined.

Surface sterilized axillary buds were inoculated on multiplication media (30ml) supplemented with sucrose (0,1,2,3 and 4%).

Results recorded (Fig.35) indicate that the cultured bud remained green for two weeks and subsequently died in absence of sucrose. Addition of sucrose to the medium at 1% level resulted in growth of the original bud into a short shoot-bud. Increasing sucrose level to 2% resulted in sprouting of 3±0.95 shoot-buds per explant. However, 5±0.50 shoot-buds

Fig. 35 Effect of sucrose
(a) 0%
(b) 1%
(c) 2%
(d) 3%
(e) 4%

on axillary bud sprouting

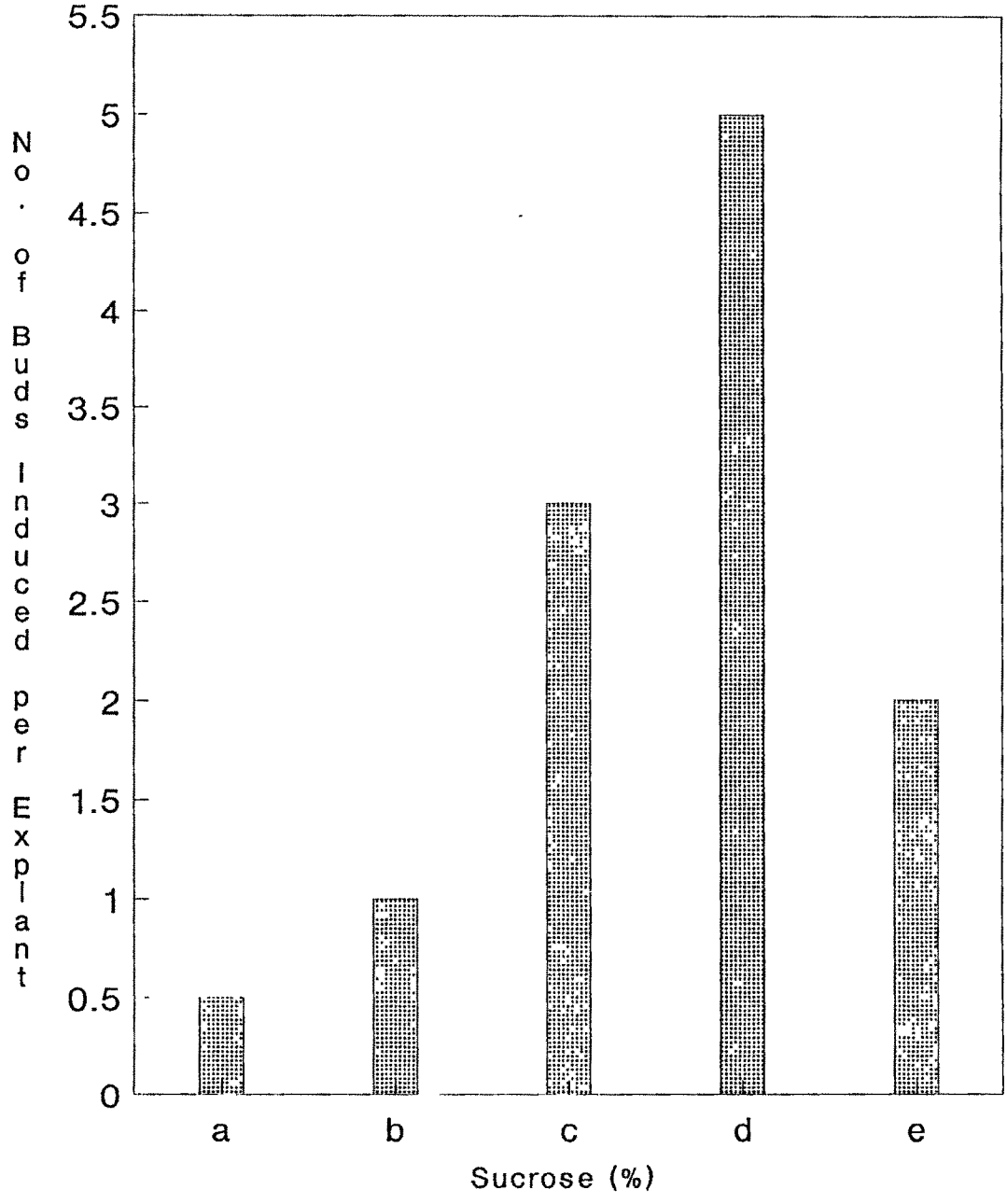

No. of Buds Induced per Explant
5.5
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
a
b
c
d
e
Sucrose (%)

Fig. 35

were produced from a single axillary bud at 3% sucrose level in 80% of the cultures. This was the maximum number of shoot-buds sprouted per explant as further increase in sucrose level to 4% resulted in a decline in the number of shoot-buds sprouted to 2±0.05 buds per explant.

Thus, sucrose at 3% provided the buds with requisite doses of energy for their maximum proliferation.

Experiment 21 : Ideal period during the year for optimal axillary bud sprouting

In order to find out the ideal period-month of the year when maximum number of shoot-buds would be produced from a single axillary bud cultured on multiplication medium, the present experiment was conducted.

Axillary buds were inoculated on multiplication media(30ml) and culture flasks were incubated in a culture room at 25±2°C in 16h photoperiod (1000 lux). Experiment was conducted bimontly *viz.* (a) Jan.-Feb. (b) March-April (c) May-

June (d) July-Aug. (e) Sept.-Oct. (f) Nov.-Dec. Results noted after four weeks were recorded in Table XVI. Maximum-minimum temperatures and relative humidity during these months were also recorded.

Axillary buds when cultured in the months of Jan.-Feb. on MS multiplication medium failed to sprout. The average maximum and minimum temperatures during these months were 30.1°C and 18.5°C respectively with relative humidity 25.6 as shown in Table XVI. During March-April i.e. spring season,when the level of endogenous growth regulator rises, the cultured axillary bud produced five shoot-buds. Besides, there was rise in maximum and minimum temperatures recorded to 33.0°C and 21.4°C respectively with simultaneous increase in relative humidity to 27.5. With the rise in temperature during the months of May-June there was a decline in the number of buds sprouted to 4 (Table XVI). July and August being the usual rainy season, endogenous contaminants in the explant posed greatest problem, resulting in death of the cultures. Still further, Sept-Oct. being autumn months when new sprouts appeared,

TABLE XVI : Ideal month for axillary bud sprouting

Medium . Multiplication medium

Inoculum : An axillary bud

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

Sr. No.	Months	Average Temperature(°C) Max.	Min.	Average Relative humidity	No.of shoot-buds/explant
1	Jan..-Feb.	30.1	18.5	25 6	1
2	March-April	33 0	21.4	27.5	5
3	May-June	37.8	27 4	40.9	4
4	July-Aug.	32 2	26 0	67.8	-
5	Sept.-Oct	33.5	24.5	58.6	4
6	Nov.-Dec.	30.5	16.8	39.0	1

Mean of six replicates

axillary bud sprouted into four buds per explant. Subsequently, in the months of Nov.-Dec. there were no fresh sprouts on the plant hence, when the experiment was conducted from the buds of the previous months, they failed to sprout.

From the above results it is evident that axillary buds produced from fresh sprouts in the months of March-April, when cultured on multiplication medium produced highest number of shoot-buds. These shootbuds - the vegetative propagules - need to be elongated to suitable length before rooting. Hence, further experiment was conducted to achieve this objective.

II. ADVENTITIOUS BUD INDUCTION

Buds induced from other positions than the normal pre existing meristematic regions, the leaf axil/apex of a branch are termed as adventitious buds. Rapid multiplication and propagation of *A.lebbeck* was achieved by adventitious bud induction from cultured leaflets.

This section deals with the standardisation of a protocol for induction of adventitious buds from leaflets and further development of these buds into plantlets.

Experiment 22 : Induction of adventitious buds in *A.lebbeck* in response to cytokinin/s

Cytokinin/s are required to activate localized meristematic tissues leading to primordium differentiation at sites other than bud meristems. Thus for adventitious bud induction, the effect of cytokinin/s, either singly or in combination was tested.

a) Effect of Kn

MS basal medium containing sucrose (3%) was supplemented with Kn (0.5 to 10µM/l). Young leaflets from selected 'elite' *A.lebbeck* were cultured following the standard procedures.

Results recorded in Table XVII indicate that there was swelling of leaflets in 0.5/1µM/l of Kn. Increasing its level to

TABLE XVII : Effect of cytokinin/s on adventitious bud induction from leaflets
Medium : MS + Sucrose (3%) + Kn /BAP/ Kn + BAP/ Kn BAP + $AdSO_4$
Inoculum : A leaflet
Incubation : Four weeks at 25±2 °C in 16h photoperiod (1000 lux)

Sr. No.	Cytokinin/s levels (µM/l)	Morphogenic response	No. of buds/ explant	% response
	Kn			
1	0.5	swelling	-	-
2	1	"	-	-
3	2	"	-	-
4	4	callusing	-	-
5	8	"	-	-
6	10	"	-	-
	BAP			
7	0 5	Curling	-	-
8	1	"	-	-
9	2	"	-	-
10	4	Shoot-buds	1	50
11	8	Shoot-buds	4 ± 0.75	70
12	10	Callusing	-	-
	Kn + BAP			
13	2 2	Curling	-	-
14	2 4	Shoot-buds	3 ± 0.10	60
15	2 8	Shoot-buds	6 ± 0.25	80
16	4 2	Curling	-	-
17	4 4	"	-	-
18	4 8	"	-	-
19	8 2	"	-	-
20	8 4	Callusing	-	-
21	8 8	"	-	-
	[Kn (2µM/l)+ BAP(8µM/l)] +$AdSO_4$			
22	2	Shoot-buds	2 ± 0.25	15
23	4	Shoot-buds	5 ± 0.30	60
24	8	Callusing	-	-

Mean of six replicates with standard deviation
- No response

2μM/l resulted in considerable swelling of leaflets to twice its size without any sign of shoot-bud initiation. Further, increase in Kn level to 4/8/10μM/l resulted in an increase in volume of both the lamina and midvein regions with the formation of pale non-chlorophyllous callus near the margins of leaflets.

b) Effect of BAP

In order to induce adventitious buds from leaflets, they were cultured on MS medium containing sucrose (3%), supplemented with BAP (0.5 to 10 μM/l) following the standard culture procedures.

Results recorded in Table XVII indicate that BAP at 0.5/1/2μM/l showed no sign of shoot-bud initiation Leaflet cultured on BAP (4μM/l), induced a single shoot-bud in the form of a greenish protuberance during 15 days incubation period, which organised itself into a shoot-bud at the end of four weeks (Fig.36a). Further increase in BAP level to 8μM/l, cultured leaflet produced 4 ± 0.75 shoot-buds (70%) as seen in Fig.36b.

Fig.36 Leaflet cultured on MS + Sucrose (3%)

a) + BAP (4μM/l)

b) + BAP (8μM/l)

c) + Kn (2μM/l) + BAP (8 μM/l)

Culture period : four weeks

a

b

c

Fig. 36

However, callus was induced from the margins of leaflets in 10μM/l of BAP.

Thus, from the above results it is evident that out of the levels tested, BAP(8μM/l) induced maximum number of shoot-buds.

c) Effect of Kn in combination with BAP

In order to enhance the number of shoot-buds induced from a leaflet, the effect of Kn in combination with BAP was tested.

MS medium containing sucrose (3%) was supplemented with Kn(2,4 and 8μM/l) and BAP(2,4 and 8μM/l). Surface sterilized leaflets were cultured on the above mentioned media and cultures were incubated following the standard procedures.

Results recorded in Table XVII indicate that in media supplemented with Kn (2μM/l)and BAP (2μM/l) there was

curling of cultured leaflets without any shoot-bud differentiation. Keeping Kn level constant and increase in BAP level to 4µM/l there was swelling of leaflets with the subsequent induction of 3±0.10 shoot-buds while at 8µM/l of BAP, cultured lealflet produced 6±0.25 shoot-buds (80%)as seen in Fig.36 c.

Increase in Kn level to 4µM/l in combination with BAP (2,4 and 8µM/l) there was initial curling of leaflets with their subsequent swelling in all the combinations tested.

Further, increase in Kn level to 8µM/l in combination with BAP (2µM/l) resulted in failure of the leaflets to show any response. Keeping Kn level constant, when BAP was increased to 4/8µM/l resulted in callus induction from the margins of leaflets.

Thus, in media supplemented with Kn (2µM/l)and BAP (8µM/l) leaflets induced optimal number of shoot-buds per explant.

d) Effect of Kn and BAP in combination with $AdSO_4$

Surface sterilized leaflets from 'elite' *A.lebbeck* were cultured on MS media containing sucrose(3%),supplemented with Kn(2µM/l), BAP(8µM/l) in combination with $AdSO_4$ (2,4 and 8µM/l). Standard culture procedures were followed.

Results recorded in Table XVII indicate that after two weeks there was swelling of leaflet which subsequently produced 2±0.25 buds in 2µM/l of $AdSO_4$ at the end of four weeks. Increase in $AdSO_4$ to 4µM/l resulted in induction of 5 ± 0.30 shoot-buds (60%). Further increase in $AdSO_4$ to 8µM/l resulted in callusing of leaflet all over its margins.

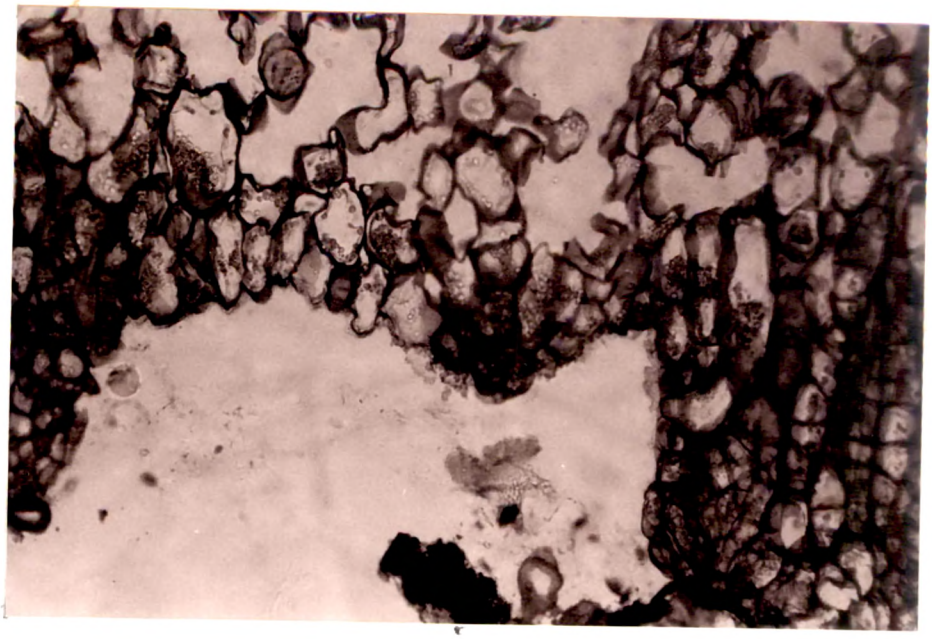
From the results of this experiment it was concluded that leaflet cultured on Kn (2µM/l) and BAP (8µM/l) induced optimal number of shoot-buds.

Histological studies during adventitious bud induction revealed that cells of lower epidermis swelled (Fig.37a) in 6 days incubation and subsequently, there was

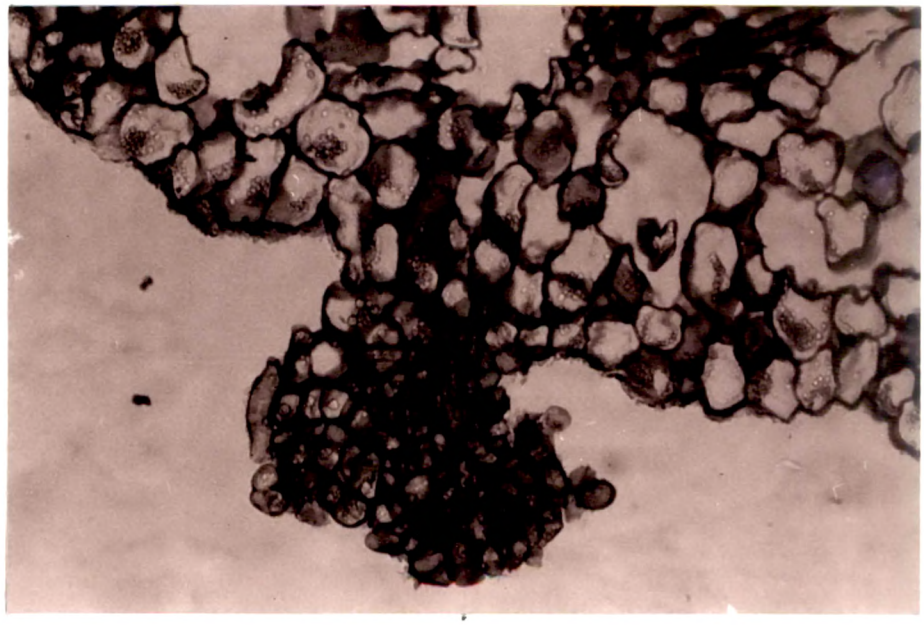
Fig. 37 T.S. of cultured leaflet showing adventitious bud induction (16X x 10X)

a) After one week

b) After four weeks



a



b

Fig. 37

bursting of the epidermis producing meristematic growth centre from parenchyma cells of the mesophyll. These meristematic growth centres organised themselves into shoot-buds (Fig.37b) in four weeks incubation.

Experiment 23 : Effect of auxin (IAA / NAA) in combination with Kn and BAP on adventitious bud induction

The effect of addition of auxin (IAA/NAA) in combination with optimal cytokinins on adventitious bud induction was tested.

MS media (30 ml) with sucrose (3%) containing Kn (2µM/l) and BAP (8µM/l) were supplemented with IAA/NAA at various levels (0.1 0.5 and 1.0µM/l). Leaflets were cultured on the above mentioned media and incubated at 25±2°C in 16h photoperiod (1000 lux) in a culture room.

Results recorded (Table XVIII) indicate that incorporation of IAA at 0.1µM/l in the medium induced 4±0.15

TABLE XVIII : Effect of auxin/s in combination with cytokinins on adventitious bud induction

Medium : MS + Sucrose (3%) + Kn (2 µM/l) + BAP (8 µM/l) + IAA / NAA

Inoculum : A leaflet

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

Sr. No.	Auxin levels (µM/l)	Number of shoot-buds induced / explant	% response
1.	Control-Kn (2µM/l) + BAP (8µM/l)	6 ± 0.25	80
	IAA		
1	0 1	4 ± 0.15	60
2	0.5	3 ± 0.25+C	55
3	1	C	50
	NAA		
4	0 1	2 ± 0 45+C	60
5	0 5	C	50
6	1	"	50

Mean of six replicates with standard deviation
C -callus

buds per explant. With the increase in IAA level to 0.5μM/l there was decrease in the number of buds induced per explant to 3±0.25 buds with basal callusing. Further increase in IAA level to 1.0μM/l resulted in leaflets producing callus without any shoot-bud differentiation.

Replacing IAA with NAA (0.1μM/l) resulted in cultured leaflets producing 2±0.45 shoot-buds with profuse callus at its base . Leaflets cultured on NAA at 0.5/1.0μM/l failed to induce shoot-buds, instead callusing occured from its margins.

Thus, from the above mentioned results it was evident that exogenous application of IAA/NAA failed to enhance the number of shoot buds per leaflet.

Experiment 24 : Effect of explant size and orientation on adventitious bud induction

Adventitious bud induction from leaflets is also dependent on its size and orientation on the medium in addition

to the effect of cytokinin/s /auxin. Hence, in this experiment, the effect of above mentioned parameters on adventitious bud induction was studied.

MS medium containing sucrose (3%) supplemented with Kn (2µM/l) and BAP (8µM/l)was inoculated with entire leaflet on abaxial/adaxial side in contact with the medium. Culture flasks were incubated following similar culture procedures. It was observed that 6±0.20 shoot-buds per explant (80%) were produced from adaxial side of the leaflet. While leaflets cultured on adaxial side in contact with the medium failed to show any response. Hence, further experiment was conducted by culturing leaflet pieces *viz.*, basal/middle/apical as well as complete leaflets on their abaxial side in contact with the above mentioned medium.

Results recorded (Table XIX) indicate that none of the leaflet pieces had the capacity for shoot bud induction, instead callusing occured from cut end, while 80% of the entire leaflet produced 6±0.20 shoot-buds. This clearly proved that

TABLE XIX : Effect of leaflet size on adventitious bud induction

Medium : MS + Sucrose (3%) + Kn (2µM/l) + BAP (8µM/l)

Inoculum : Excised/ entire leaflet

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

Sr. No.	Leaflet size	No. of buds induced/explant	% response
1	Excised		
	Basal	-	-
	Middle	-	-
	Apical	-	-
2	Entire	6 ± 0.20	80

Mean of six replicates with standard deviation

leaflet cultured on abaxial side in contact with medium possessed bud inducing capacity.

III. PLANT REGENERATION FROM CALLUS CULTURES

Experimental work was conducted on the establishment of callus cultures of stem/leaflet of 'elite' *A.lebbeck* plant in order to regenerate plantlets from them. Murashige and Skoog's (1962) medium containing sucrose (2%) with appropriate doses of phytohormones was used to initiate callus from both the cultured explants. Subsequently, nutritional and hormonal levels for achieving highest biomass values in terms of fresh and dry weights of both the callus tissues were standardised. Further, the ability of these callus tissues for synthesis/accumulation of quercetin was evaluated. Finally, the morphogenic potential of callus tissues was also examined.

Experiment 25 : Callus induction from stem/leaflet pieces of 'elite' *A.lebbeck* plant

M$ medium (30 ml) containing sucrose (2%) was supplemented with Kn/BAP (0,1,2 and 4µM/l) in combination

with IAA/NAA/ 2,4-D (0,1,2,4,8 and 10μM/l), adjusting the pH to 5.8 before gelling with agar (0.8%).

Healthy stem/leaflet pieces were excised from 'elite' *A.lebbeck* and after following the culture procedures as described in Chapter II were inoculated on the above mentioned media and incubated in culture room for 16h photoperiod (1000 lux) light intensity. Six replicates per treatment were maintained.

Results recorded in Table XX after four weeks indicate that stem pieces when cultured on basal MS medium containing sucrose (2%) in absence of Kn turned brown while they remained green without any sign of callus induction on media supplemented with Kn (1,2 and 4μM/l). Replacing Kn with BAP at the same levels in the culture media, cultured stem pieces failed to show callus induction. Stem explants on BAP supplemented media remained green for one week and then turned black, proving the inability of BAP in inducing callus.

TABLE XX : **Callus induction from stem segments/ leaflets of *A.lebbeck Benth*.**

Medium : MS + Sucrose (2%) + BAP/ Kn / (Kn + IAA/ NAA / 2,4-D)

Inoculum : Stem segments/ leaflets

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

Sr. No.	Auxin (µM/l)		Stem segments				Leaflets			
		BAP (1-4µM/l)	-				-			
		Kn (µM/l)	0	1	2	4	0	1	2	4
1	Control		-	-	-	-	-	-	-	-
	IAA									
2	1		-	-	-	-	-	-	-	-
3	2		-	-	-	-	-	-	-	-
4	4		-	+	+	+	-	+	+	+
5	8		-	+	+	+	-	+	+	+
6	10		-	+	+	+	-	+	+	+
	NAA									
7	1		-	-	-	-	-	-	-	-
8	2		-	-	-	-	-	-	-	-
9	4		-	-	-	-	-	-	-	-
10	8		-	-	-	-	-	-	-	-
11	10		-	-	-	-	-	-	-	-
	2,4-D									
12	1		-	++	++	++	-	++	++	++
13	2		-	++	++	++	-	++	++	++
14	4		-	++	++	++	-	++	++	++
15	8		-	+++	++++	+++	-	+++	++++	+++
16	10		-	+++	++	++	-	+++	++	++

Mean of six replicates
- No response
+ Amount of callus induced

Leaflet explants cultured on basal medium failed to show any response and subsequently died. In Kn(1,2and 4μM/l) supplemented media there was swelling and subsequent curling of leaflets without any callus induction while in BAP(1,2and 4μM/l) supplemented media, leaflets turned fleshy without any sign of callus induction.

Hence, among the cytokinins tested, Kn was chosen for using it in combination with auxins (IAA/NAA/2,4-D)in order to test their ability for callus initiation from explants. Stem explants when cultured on Kn (1μM/l) in combination with IAA (1 and 2μM/l) failed to show callus induction. Keeping IAA level constant,when Kn was increased to 2/4μM/l, cultured stem explant failed to show callus induction. Further, stem explants when cultured on Kn at 1,2 and 4μM/l in combination with IAA (4,8 and 10μM/l) resulted in induction of white coloured callus in four weeks. This callus was hard and compact.

Leaflet explants cultured on Kn (1μM/l) in combination with IAA (1 and 2μM/l) showed their swelling

without any sign of callus induction. Keeping IAA level constant, when Kn was increased to 2/4μM/l, cultured leaflet explant remained without any response. Increasing IAA level to 4,8 and 10μM/l in combination with Kn(1,2 and 4μM/l), leaflet pieces induced light yellow coloured compact callus in four weeks .

Keeping Kn levels constant and replacing IAA with NAA at the above mentioned levels, both explants failed to show callus induction.

Stem pieces when cultured on Kn (1μM/l) with 2,4-D (1,2 and 4μM/l) showed callus initiation from their cut ends in three weeks. Keeping Kn level constant when 2,4-D level was increased to 8/10μM/l, cultured stem explant showed swelling and finally induction of callus in two weeks. Increasing Kn level to 2 and 4μM/l in combination with 1,2 and 4μM/l of 2,4-D resulted in stem explants producing friable callus in three weeks. Keeping Kn level as 2μM/l and increasing 2,4-D level to 8μM/l, cultured stem pieces produced greenish yellow friable

callus in one week which covered the explant in four weeks (Fig.38a). Maintaining Kn level constant and increasing 2,4-D level to 10μM/l resulted in callus initiation from stem explant in four weeks.

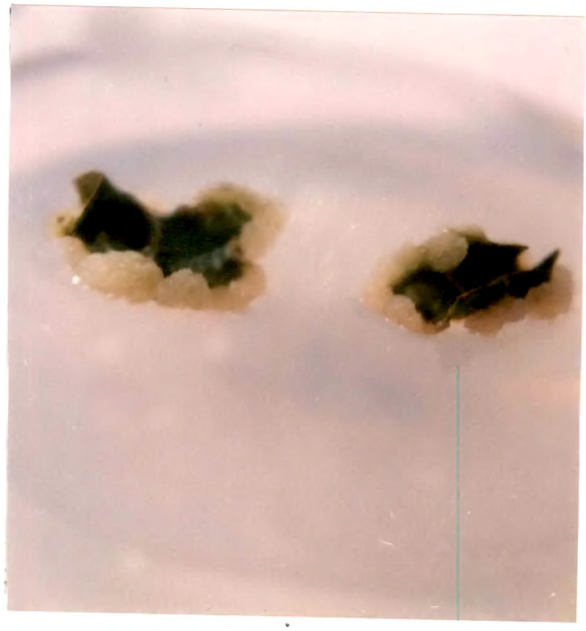
Leaflets cultured on media supplemented with Kn (1μM/l) in combination with 2,4-D (1,2 and 4μM/l) induced callus in three weeks incubation. Keeping Kn level constant and increasing 2,4-D level to 8/10μM/l resulted in callus initiation in two weeks. At Kn(2/4μM/l) in combination with 2,4-D (1,2and 4μM/l), leaflet explants induced friable callus in a period of three weeks while on Kn (2μM/l) with 2,4-D (8μM/l) supplemented media, cream coloured friable callus was induced in one week which proliferated over its surface in four weeks (Fig.38b). Further, increase in 2,4-D level to 10μM/l resulted in induction of callus at a slower rate. At Kn (4μM/l) in combination with 2,4-D (1,2 and 4μM/l) cultured leaflet explant induced friable callus in three weeks while at 8μM/l of 2,4-D, rate of callus induction was increased, taking about two weeks

Fig. 38 Callus induced from
a) Stem pieces

b) Leaflet pieces

culture period - four weeks

a



b

Fig. 38

for its initiation. Further, increase in 2,4-D level to 10μM/l resulted in callus initiation in four weeks.

Thus, out of the hormone levels tested, Kn (2μM/l) in combination with 2,4-D (8μM/l) proved to be the optimal levels for callus initiation from stem/leaflet explants.

Experiment 26 : Optimal biomass production ofcallus cultures

Appropriate nutritional and hormonal levels were to be standardised to achieve optimal biomass production of stem and leaflet callus cultures. These callus tissues were subsequently used to study their morphogenetic and biosynthetic potentials.

a) Efect of various carbohydrates

For sustained proliferation of callus tissues there was a necessity of suitable carbon source. Hence, the present experiment was conducted to determine the effect of various carbohydrates on the growth of callus tissues.

Healthy, fast growing stem/leaflet callus tissues (300±20mg, fr.wt. and 20±01mg, dry wt.) were cultured on MS medium containing Kn(2μM/l) with 2,4-D (8μM/l) and supplemented with monosaccharide *viz.*,glucose/discaccharide *viz.*, sucrose/polysaccharide *viz.*, mannitol(2%).

Results depicted in Fig.39 indicate that stem callus when cultured on glucose (2%) supplemented media produced a biomass of 2500±20mg and 165±05mg in terms of fresh and dry weights respectively. This callus was hard and compact. While stem callus when grown on sucrose (2%) supplemented media reached to a biomass of 3150±22mg, fr. wt. and 205±03mg, dry wt. This callus tissue was friable. Least amount of callus growth occured in mannitol (2%) supplemented media (Fig.39).

Leaflet callus when cultured on glucose (2%) supplemented media reached to a biomass of 2650±25mg and 170±02mg as fresh and dry weights respectively. Replacing glucose with sucrose at the same level resulted in a considerable increase in fresh and dry weights to 3750±15mg

Fig. 39 Growth of stem/leaflet callus tissues on MS medium containing Kn (2μ M/l) and 2, 4-D (8μ M/l) supplemented with (2%)

a) Glucose
b) Sucrose
c) Mannitol

culture period - four weeks

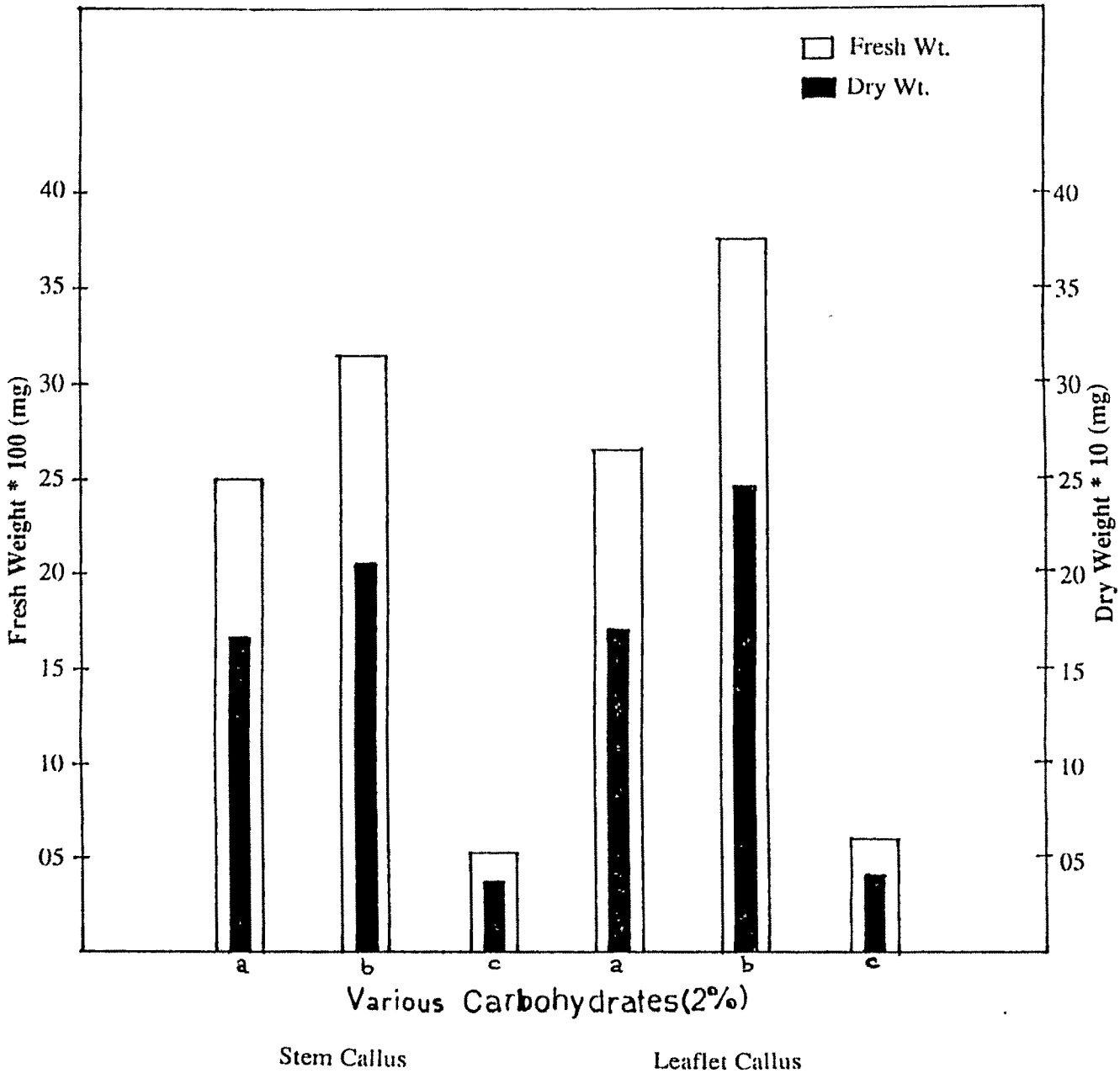

Fresh Wt.
Dry Wt.
Fresh Weight * 100 (mg)
Dry Weight * 10 (mg)
40
35
30
25
20
15
10
05
a
b
c
a
b
c
Various Carbohydrates(2%)
Stem Callus
Leaflet Callus

Fig. 39

and 245±01mg respectively. In comparison to glucose supplemented media, sucrose supplemented media produced friable callus. Mannitol at 2% recorded poor growth of the callus tissue as seen in Fig.39

Thus, it was concluded that sucrose at 2% supported maximum growth of stem and leaflet callus tissues. Hence, sucrose proved to be superior over other carbohydrates tested. Still, the level of sucrose required for optimal biomass of callus cultures remained to be determined.

Healthy stem/leaflet callus tissues (300±20mg, fr.wt. and 20±02mg, dry wt.) were inoculated on MS media (30ml) containing Kn(2µM/l) and 2,4-D (8µM/l), supplemented with sucrose at 0,1,2,3 and 4%. Culture flasks were incubated as usual.

Results recorded at the end of four weeks as depicted in Fig.40 indicate that stem callus cultures failed to grow on the medium in absence of sucrose while at its 1% level

biomass value reached to 1500± 10mg and 100±02mg in terms of fresh and dry weights respectively. Increase in sucrose level to 2% resulted in two fold increase in biomass (fr.wt., 3200±15mg and dry wt., 200±05mg). This was the optimal level of sucrose as its further increase to 3 and 4% resulted in a decline in growth of the callus tissues (Fig.40).

Leaflet callus cultured in absence of sucrose showed negligible growth (Fig.40). Addition of sucrose at 1% induced fast growth of callus with fresh weight,1600±15mg and dry weight, 105±03 mg. Increase in sucrose level to 2% induced optimal callus growth (fr.wt. 3700±10mg & dry wt. 230± 01mg)as there was a decline in biomass values at 3% and 4% sucrose levels (Fig.40).

Thus, it was concluded that sucrose at 2% proved to be the optimal level for biomass production of both the callus tissues.

b) Effect of Kn levels

Fig. 40 Growth of stem/leaflet callus tissues on
MS + Kn (2μ M/l) + 2, 4-D (8μ M/l) + Sucrose (0,1,2,3 and 4%)

culture period : Four weeks

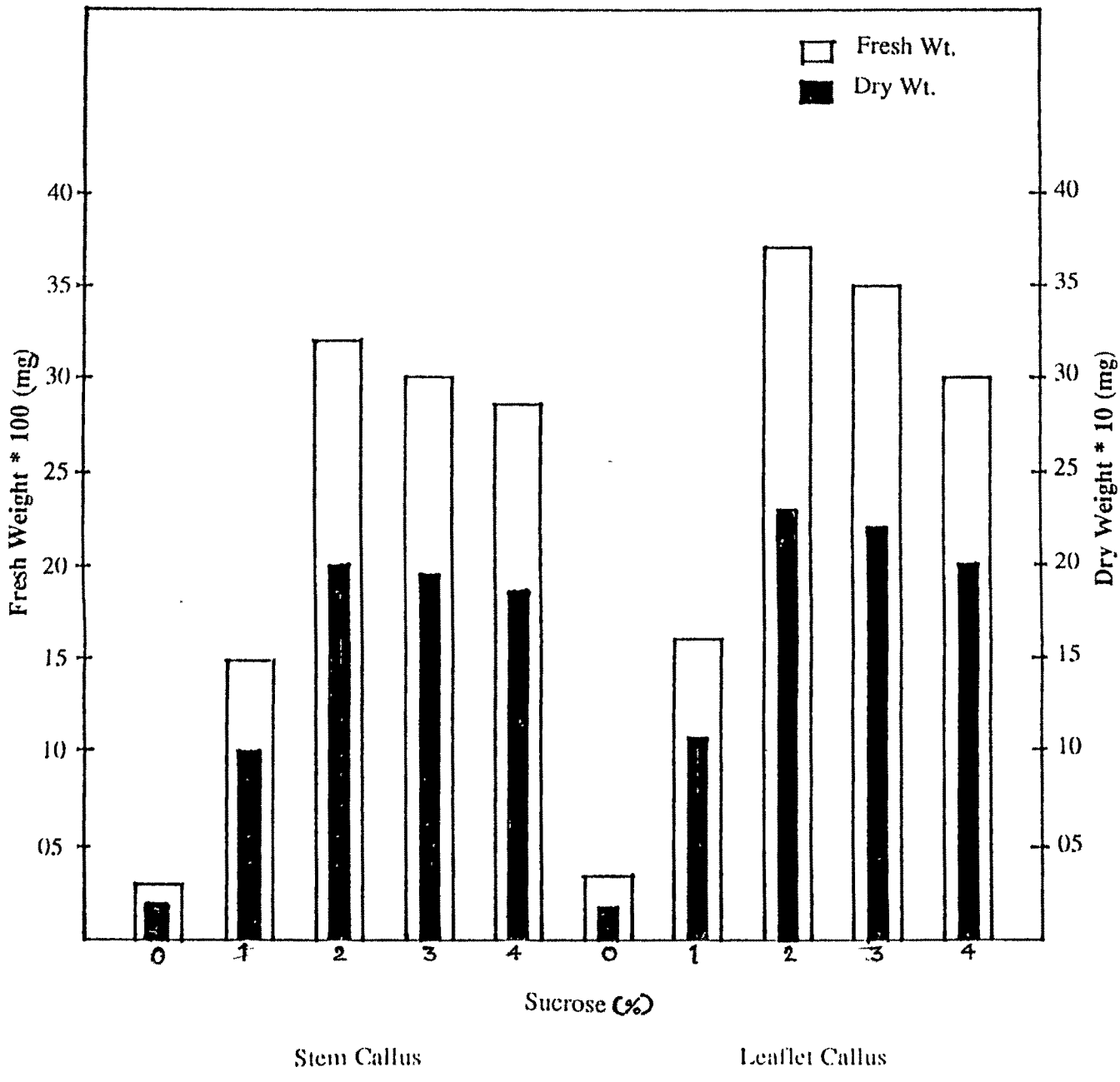

Fresh Wt.
Dry Wt.
Fresh Weight * 100 (mg)
Dry Weight * 10 (mg)
40
35
30
25
20
15
10
05
0
1
2
3
4
Sucrose (%)
Stem Callus
Leaflet Callus

Fig. 40

MS media (30ml) containing sucrose (2%), 2,4-D at 8μM/l supplemented with Kn (0,1,2 and 4μM/l) and inoculated with healthy, fast growing stem and leaflet callus tissues (300 ± 20mg, fr. wt. and 20±05mg,dry wt.). Experimental procedures were followed as described in Chapter II, Materials and Methods.

Results presented in Fig.41 indicate that in absence of Kn,stem callus tissues registered slight biomass production 550±20mg and 30±01mg in terms of fresh and dry weights respectively. Supplementing Kn(1μM/l) to the basal medium induced considerable enhancement of stem callus biomass to 2100±20mg and 135±02mg in terms fresh and dry weights respectively.With the increase in Kn level to 2μM/l, stem callus proliferated at a faster rate attaining a fresh weight of 3200±25mg and dry weight of 215±05mg. These were the maximum biomass values recorded as further increase in Kn level to 4μM/l reduced fresh and dry weights of the callus tissue (Fig.41).

Fig.41 Growth of stem/leaflet callus tissues on
MS + Sucrose (2%) + 2, 4 - D (8μM/l) + Kn(0 ,1,2 and 4μM/l)

culture period - four weeks

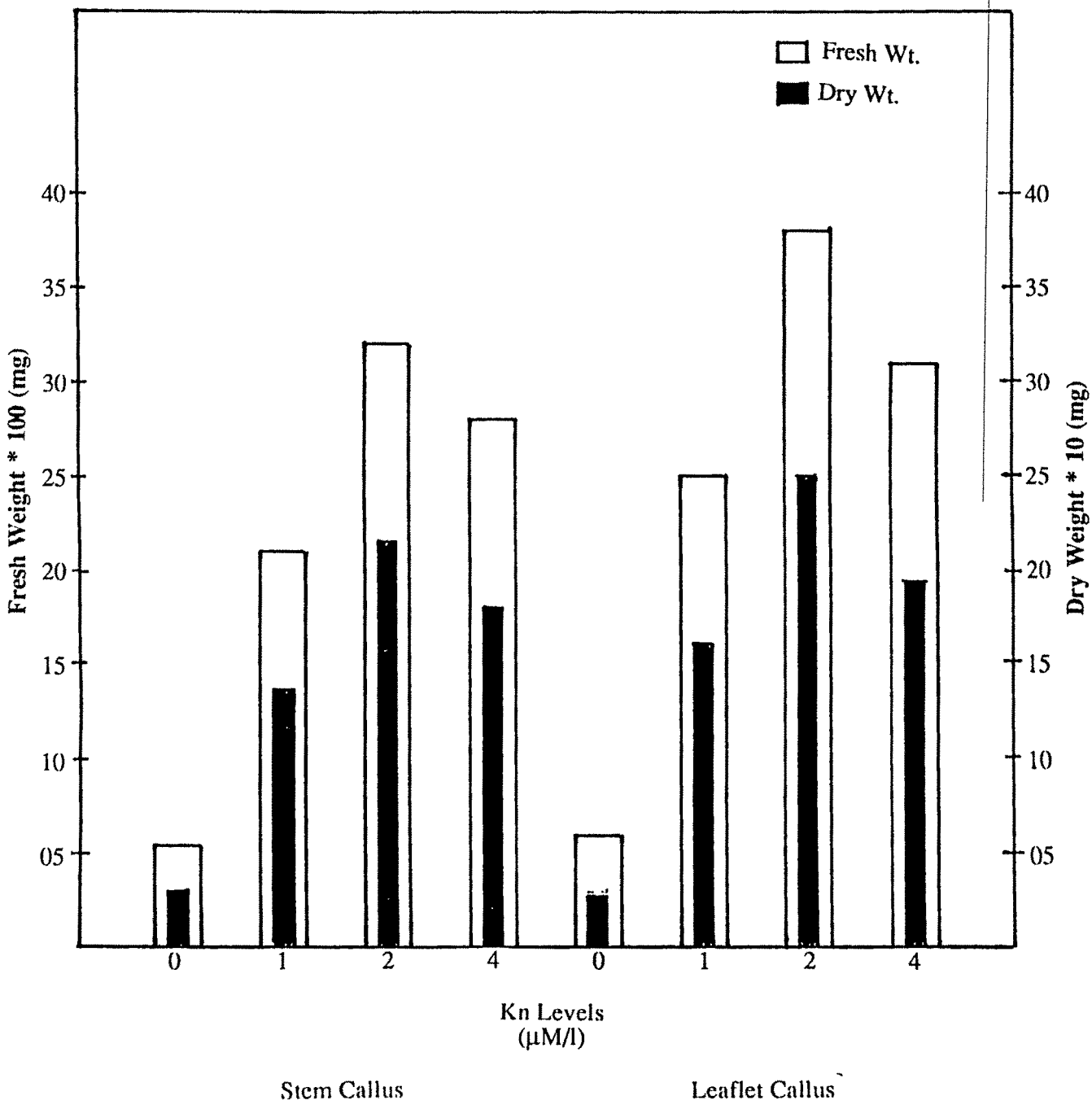

Fresh Wt.
Dry Wt.
Fresh Weight * 100 (mg)
Dry Weight * 10 (mg)
40
35
30
25
20
15
10
05
0
1
2
4
0
1
2
4
Kn Levels
(μM/l)
Stem Callus
Leaflet Callus

Fig. 41

Leaflet callus cultured on medium lacking Kn induced 600±10mg and 30±05mg biomass in terms of fresh and dry weights respectively after four weeks incubation period. Supplementing the medium with Kn (1µM/l), leaflet callus proliferated at a rapid rate acquiring biomass, 2500±50mg and 160±05mg in terms of fresh and dry weights respectively. Increase in Kn level to 2µM/l induced considerable enhancement in biomass values to 3800±28mg and 250±02mg in terms of fresh and dry weights respectively (Fig.41). These were the maximum biomass values as further increase in Kn level to 4µM/l resulted in a decline in biomass of leaflet callus tissue to 3100±10mg and 195±01mg in terms of fresh and dry weights respectively.

Thus, it was evident that Kn (2µM/l) was optimal for achieving highest biomass of both the callus tissues.

c) Effect of 2,4-D levels

Healthy, fast growing stem and leaflet callus tissues, weighing 300±20mg by fresh weight were cultured on

MS media (30ml) containing sucrose (2%), Kn (2μM/l) were supplemented with 2,4-D (0,1,2,4,8 and 10μM/l).

Results presented in Fig.42 indicate that in absence of 2,4-D, there was very little growth of stem callus in terms of fresh and dry weights to 600±18mg and 42±02mg respectively. Addition of 2,4-D at 1μM/l resulted in an increase in biomass value to 1250±10mg and 80±01mg in terms of fresh and dry weights. These biomass values reached to their maximum,3300±10mg and 220±02mg in terms of fresh and dry weights respectively, when 2,4-D level was 8μM/l. Further increase in 2,4-D level to 10μM/l resulted in a decline in biomass values (Fig.42).

Leaflet callus (300±20mg,fr.wt. and 20±01mg,dry wt.) when cultured on medium lacking 2,4-D recorded 685±20mg, fr.wt. and 48±05mg, dry wt. Supplementing 2,4-D(1μM/l) to MS medium resulted in enhancement of biomass to 1325±40mg and 85±01mg as fresh and dry weights respectively. This biomass value increased with the increase in 2,4-D level,

Fig. 42 Growth of callus tissues of stem/leaflet on
MS + Sucrose (2%) + Kn(2μ M/l) + 2, 4-D (0 ,1,2,4,8 and 10μM/l)

culture period - four weeks

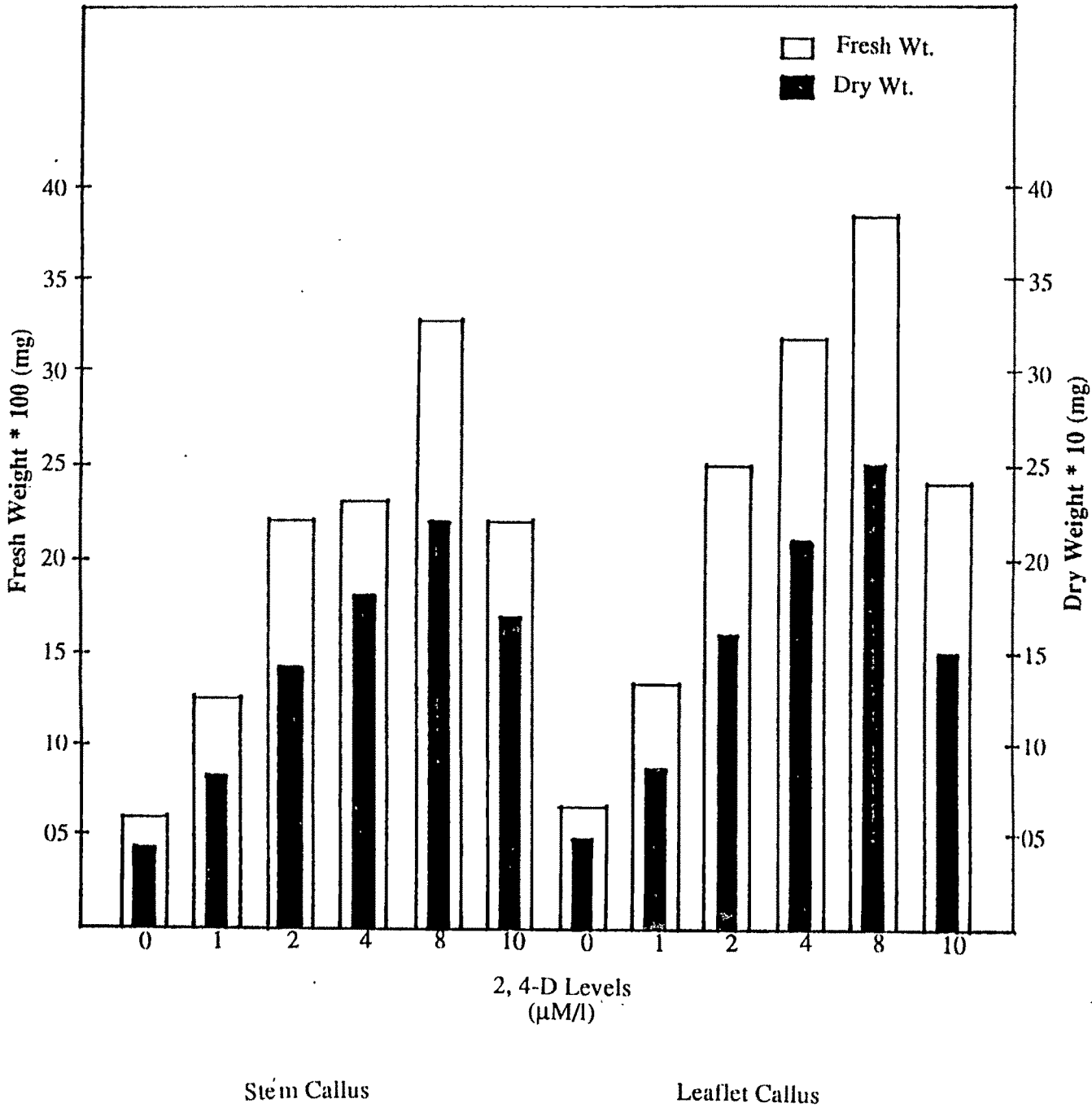

Fresh Wt.
Dry Wt.
Fresh Weight * 100 (mg)
Dry Weight * 10 (mg)
40
35
30
25
20
15
10
05
0
1
2
4
8
10
2, 4-D Levels
(µM/l)
Stem Callus
Leaflet Callus

Fig. 42

maximum being at 8μM/l level i.e. 3850±25mg, fr.wt. and 250± 01mg, dry wt. Further increase in 2,4-D level to 10μM/l resulted in a decline in biomass values (Fig.42).

From the results of this experiment, it was concluded that MS medium containing sucrose (2%) supplemented with Kn(2μM/l) and 2,4-D(8μM/l) was designated as 'Standard medium' for callus cultures.

Experiment 27 : Quercetin profile of callus cultures

Stem and leaflet callus tissues were examined for their ability to synthesise/accumulate quercetin during eight weeks culture period.

Standard medium (30ml) for callus cultures was inoculated with 300±20mg, fresh weight of stem/leaflet callus tissues. Culture flasks were incubated following similar culture procedures.

Results presented in Fig.43 indicate a slight growth of stem callus tissue to 600±05mg and 40±01mg in terms of fresh and dry weights respectively. Biomass values of the stem callus tissue increased steadily in the second and third weeks, reaching to 1200±20mg and 70±05mg ; 3150±35mg and 200±10mg in terms of fresh and dry weights respectively. Further, at the end of four and eight weeks, biomass values reached to 3200±10mg & 210±02mg ; 3250±25mg & 215±01mg as fresh and dry weights respectively.

Chemical analysis of stem callus tissues during eight weeks indicate that quercetin was not accumulated during three weeks of culture period. At the end of four weeks, 0.005% quercetin was detected which showed no enhancement even after eight weeks.

In the leaflet callus, the biomass values in terms of fresh and dry weights were 700±10mg and 45± 02mg respectively at the end of first week. With the passage of time in the second and third weeks, two fold increase in terms of

Fig. 43 Quercetin profile of stem and leaflet callus tissues during eight weeks period

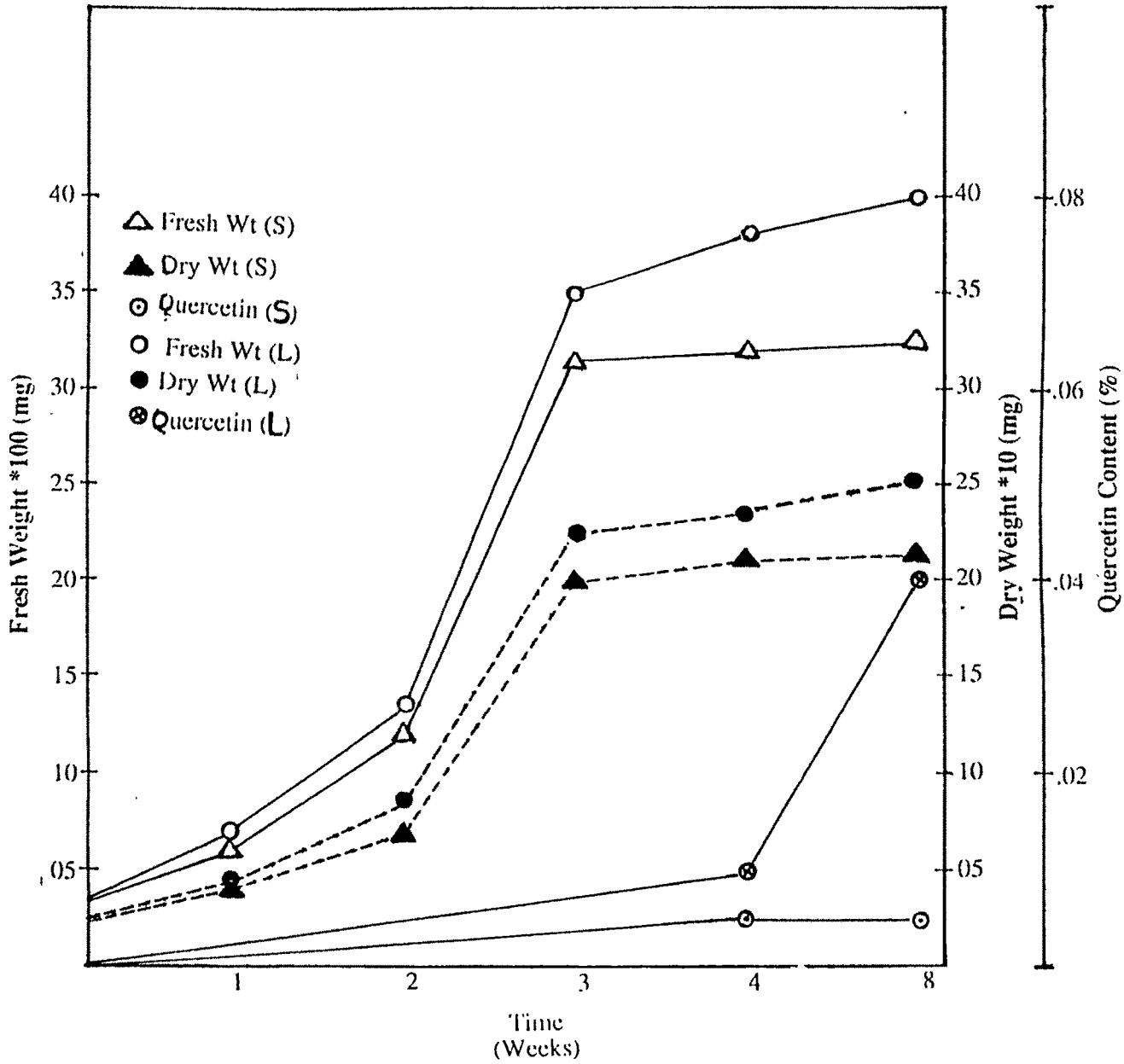

Fresh Wt (S)
Dry Wt (S)
Quercetin (S)
Fresh Wt (L)
Dry Wt (L)
Quercetin (L)
Fresh Weight *100 (mg)
Dry Weight *10 (mg)
Quercetin Content (%)
Time
(Weeks)
40
35
30
25
20
15
10
05
.08
.06
.04
.02
1
2
3
4
8

Fig.4 3

fresh and dry weights was recorded (fr.wt., 1350±20mg and 3500±35mg & dry wt., 85±01mg and 225±05mg). Further, at the end of four weeks, biomass values reached to 3800±10mg and 245±01mg in terms of fresh and dry weights respectively. Subsequently, at the end of eight weeks, fresh and dry weight values reached to 4000±50mg and 250±02mg respectively.

Quantitative estimation of quercetin from leaflet callus at weekly intervals revealed that in the first three weeks callus failed to synthesise/accumulate quercetin. While at the end of four weeks, 0.01% quercetin was detected. Subsequently, at the end of eight weeks, considerable increase in quercetin content (0.04%) was detected.

The results of this experiment proved that stem and leaflet callus cultures exhibited the capacity for synthesis/ accumulation of quercetin. Among the callus cultures tested, leaflet callus possessed better biosynthetic potential for quercetin synthesis.

Experiment 28 : Morphogenic potential of callus tissues

This experiment was conducted with an objective to regenerate plantlets from stem and leaflet callus tissues by subjecting them to various cytokinin and auxin treatments.

Healthy callus tissues from stem/leaflet grown on standard media were transferred to basal MS media for a week in order to reduce the carry over effect of the phytohormones used.

Subsequently, these callus tissues (300±20mg,fr.wt.) were transferred to MS medium (30ml) containing sucrose (3%) supplemented with Kn/ BAP used singly or in combination with IAA/ NAA/ IBA at various levels. Culture flasks were incubated in a culture room at 25±2°C in 16h photoperiod at 1000 lux light intensity.

Results depicted in Table XXI indicate that stem and leaflet callus tissues failed to show any morphogenic

TABLE XXI : Morphogenic response of callus tissues

Medium : MS+Sucrose (3%)+Kn/BAP/(BAP+IAA/NAA/IBA)

Inoculum : Healthy callus tissues (1-2 cm in dia.)

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

Sr. No.	Growth Regulators µM/l)	Morphogenic Response		% Reponse	
		Stem callus	Leaflet callus	Stem callus	Leaflet callus
1.	Kn (1- 6)	-	-	-	-
	BAP				
2.	1	Green	Green	60	60
3.	2	Green & compact	Green & compact	70	80
4.	4	Green & compact	Green & compact	80	90
5.	6	Green	Green & compact	50	50
	BAP + IAA				
6.	2 0.2	NC	NC	50	70
7.	2 0.4	NC	NC	70	80
8.	2 0.6	-	NC	-	50
	BAP + NAA				
9.	2 0.2	-	-	-	-
10.	2 0.4	-	-	-	-
11.	2 0.6	-	-	-	-
	BAP + IBA				
12.	2 0.2	-	-	-	-
13.	2 0.4	-	-	-	-
14.	2 0.6	-	-	-	-

Means of six replicates
- No response
NC Nodular callus

response in Kn supplemented media at all the levels tested while in BAP (1μM/l) supplemented media both the callus tissues turned green. Increase in BAP level to 2/4μM/l resulted in greening and compactness of the stem and leaflet callus cultures, of which leaflet callus showed better response than stem callus. Further , increase in BAP level to 6μM/l resulted in a loss of compactness of stem callus while leaflet callus remained green and compact with less number of cultures responding.

Hence, BAP at 2μM/l proved superior for inducing morphogenic response in maximum number of callus cultures. In order to differentiate shoot-buds from green and compact callus, it was subcultured on media supplemented with BAP (2μM/l) and IAA/ NAA/IBA (0.2,0.4 and 0.6μM/l). BAP (2μM/l) in combination with IAA at 0.2,0.4μM/l induced nodules in both the callus cultures, with leaflet callus cultures showing a better response. Out of these two levels, 0.4μM/l proved superior with 80% response in leaflet callus cultures (Fig.44). Further, increase in IAA level to 0.6μM/l, stem callus failed to show

Fig. 44 Green and compact leaflet callus

Fig. 45 T.S. of a nodule showing thin walled concentrically arranged embryonic cells with prominent nuclei (10X x 100X)

Fig. 44

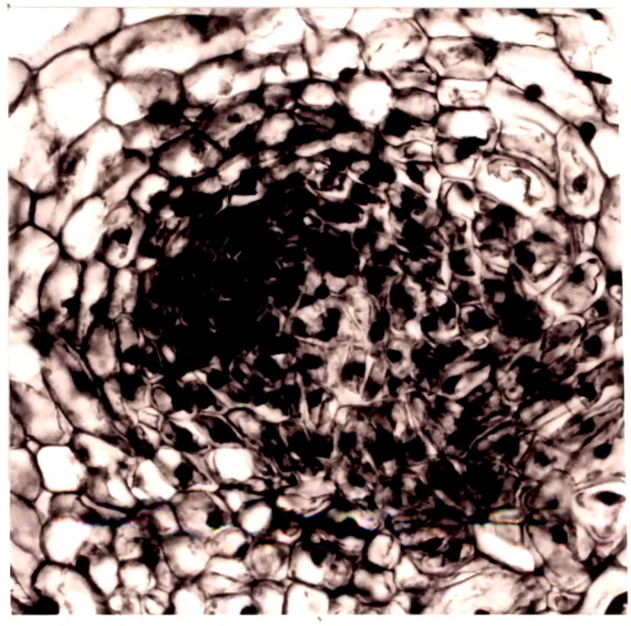


Fig. 45

any morphogenic response but leaflet callus turned nodular in 50% of the cultures.

Keeping BAP (2μM/l) level constant and replacing IAA with NAA or IBA both callus tissues failed to show any morphogenic response in all the levels tested.

Thus, from the above results it is evident that both callus cultures turned nodular in media supplemented with BAP (2μM/l) and IAA (0.4μM/l), of which leaflet callus cultures depicted better response than stem callus.

Histological observation of a single nodule from leaflet callus revealed that it consisted of meristematic growth centres formed of thin walled concentrically arranged cells with prominent nuclei (Fig. 45). In order to differentiate the nodular callus into shoot-buds it was subcultured on media containing BAP (0.1-2.0μM/l) . Results presented in Table XXII indicate that leaflet callus induced 1-2 shoot-buds in 50% cultures in 0.1μM/l of BAP while 6-7 shoot- buds differentiated at its

TABLE XXII : Shoot - bud differentiation from callus tissues

Medium : MS + Sucrose (3%) + BAP

Inoculum : Nodular leaflet callus (1-2 cm in dia.)

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

Sr. No.	BAP level (µM/l)	Number of shoot-buds differentiated from nodular callus	% response
1.	0.1	1-2	50
2.	0.5	6-7	80
3.	1.0	3-4	60
4.	2.0	-	-

Mean of six replicates

- No response

0.5μM/l level in 80% of the cultures. Increase in BAP level to 1μM/l induced 3-4 shoot-buds in 60% of the cultures (Fig.46). This level proved to be optimal for shoot-bud differentiation as further increase in BAP level reduced the number of shoot-buds differentiated (Table XXII).

Hence, BAP (1μM/l) proved to be optimal for shoot-bud differentiation from leaflet callus.

Experiment 29 : Elongation of shoot-buds

Shoot-buds produced by multiplication of axillary buds/ adventitious buds/callus cultures were subjected to GA_3 treatment in order to induce elongation in them.

Individual isolated shoot-bud, about 1-1.5cm long were transferred to MS media containing sucrose (3%),supplemented with GA_3 at various levels (0.5,1.0,2.0 and 4.0μM/l).They were maintained in the culture room following similar culture procedures.

Fig 46 Differentiation of callus into shoot-buds

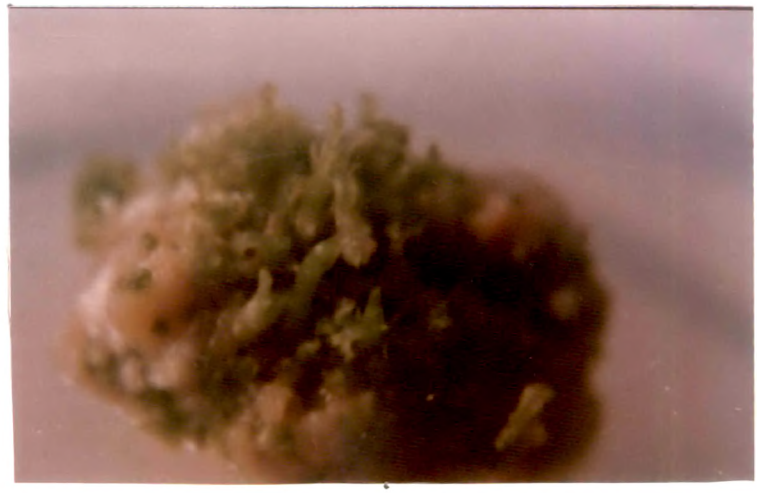


Fig. 46

From the results recorded in Table XXIII it was evident that at lower level of GA_3 (0.5µM/l) shoot-buds elongated upto the length of 5±0.21cm in 70% cultures. However, at 1µM/l of GA_3 shoot-buds reached to 10±0.05cm in length as seen in Fig.47. This was the maximum length acquired by shoots as further increase in GA_3 level proved to be unsuitable for elongation of shoot-buds (Table XXIII).

Thus, it was evident that GA_3 supplemented media induced elongation of shoot - buds, maximum being at 1µM/l level.

Experiment 30 : Plant formation

In order to induce roots from shoots, the effects of three different auxins *viz.*, IAA/IBA/NAA at various levels (0.1, 0.5, 1.0 and 2.0µM/l) were tested. Shoots(2-2.5cm long)were cultured on the above mentioned auxin supplemented half strength MS media containing sucrose (1%).

TABLE XXIII : Effect of Gibberellic acid on shoot elongation

Medium : MS + Sucrose (3%) + GA_3

Inoculum : Isolated shoot-bud

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

Sr. No.	GA_3 levels (µM/l)	Length of shoot (cm)	% response
1	0.5	5±0.21	70
2	1	10±0.05	85
3	2	2±0.10	50
4	4	2±0.05	55

Mean of six replicates with standard deviation

Fig.47 Elongation of shoot
Medium : MS + sucrose (3%)
+ GA_3 (1μM/l)

Incubation : four weeks

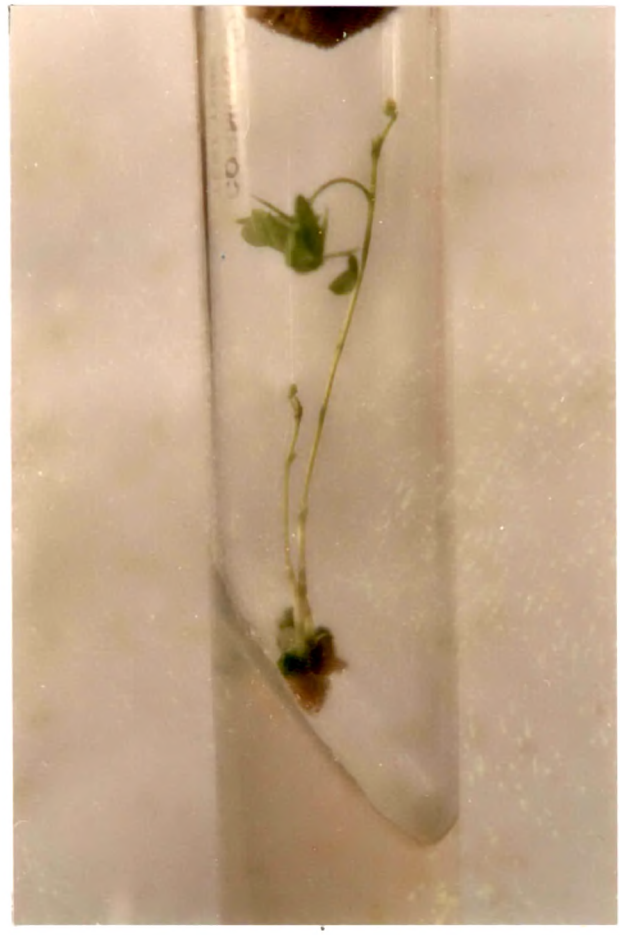


Fig. 47

Results recorded (Table XXIV) showed that IAA at 0.1μM/l induced root initial within one week which did not grow further even after four weeks. Increase in IAA level to 0.5μM/l induced tap root from the shoot in four weeks in 90% of the cultures (Fig.48). Further increase in IAA level to 1 μM/l induced very thin and delicate roots in 80% of the cultures while at 2μM/l of IAA callus was induced from cut ends of shoots.

Replacing IAA with IBA at 0.1μM/l, roots were produced with little callus at the cut end of shoot. Increase in IBA level to 0.5 μM/l resulted in induction of slow growing thick root which started callusing after three weeks. Further increase in IBA level to 1/2μM/l resulted in profuse callusing from the base of the shoot.

On NAA (0.1μM/l) supplemented media, shoots produced callus from their cut ends and then very weak hairy roots were produced. Increase in NAA level to 0.5 and 1.0μM/l cultured shoots produced roots with callus while at its 2.0μM/l shoots

TABLE XXIV : Effect of auxins on root induction

Medium : MS/2 + Sucrose (1%) + IAA/IBA/NAA

Inoculum : A well developed shoot

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

Sr. No.	Auxin level (µM/l)	Induction of roots/callus	% response
	IAA		
1	0.1	Root	70
2	0.5	Tap root	90
3	1	Root	80
4	2	C	80
	IBA		
5	0.1	Root + C	60
6	0.5	Root + C	80
7	1	C	70
8	2	C	70
	NAA		
9	0.1	Root + C	80
10	0.5	Root + C	70
11	1	Root + C	80
12	2	C	70

Mean of six replicates
C - Callus

Fig.48 Development of root from a well developed shoot in IAA (0.5µM/l)

Fig. 48

failed to induce roots but produced intensive callus from their base.

Thus, from the results of this experiment it is concluded that IAA (0.5μM/l) induced a tap root at the base of the shoot in four weeks.

Experiment 31 : Acclimatization and transfer of plants to field

Plants were transferred to pots containing sterilised vermiculite after washing the roots with sterile water. These plants were covered for one week with a glass beaker providing time to the plants for adjustment to environmental conditions (Fig.49). Subsequently, they were transferred to pots containing soil where they were watered daily. They were gradually transferred to field where they recorded 80% survival at the end of one year period.

Experiment 32 : Determination of ploidy of the regenerated plants

Fig.49 *In vitro* developed plant transferred to vermiculite and covered with glass beaker

Fig. 50 Root tip squash of the plantlet showing diploid (2n = 26) chromosomes at the metaphase plate

Fig.49

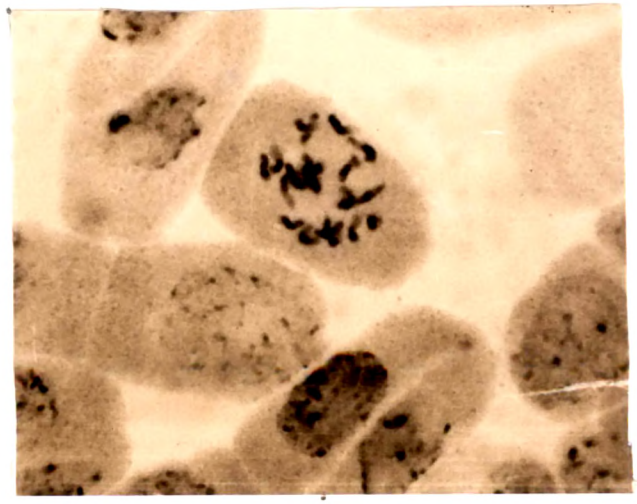


Fig. 50

In order to determine the ploidy of the regenerated plants, there root tip squash preparations were made as described in Materials and Methods, Chapter II.

Cells with metaphase plate were observed and their chromosome number was noted. All the cells revealed the presence of 2n=26 chromosomes (Fig.50). Thus, confirming the diploid nature of regenerated plants.