

CHAPTER III - CLONAL PROPAGATION OF ELITE TREES

CHAPTER III**CLONAL PROPAGATION OF ELITE TREES**

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III.

RESULTS

CLONAL PROPAGATION OF ELITE TREES

As mentioned in the introduction, one of the main objectives of the present investigation was to identify 'elite' trees growing in different agroclimatic conditions and to explore possibilities of developing a method for its clonal multiplication. Stem cuttings of trees growing in three different localities had been planted in the University Botanical Garden after giving various treatments. Attempts were also made to clonally propagate the trees by employing shoot tip culture technique.

In the preliminary trials, it was observed that the explants (axillary and terminal buds, nodal explants) were morphogenetically unresponsive, moreover, endogenous microbial contaminations were very severe. Bacteria or fungi developed either from the cut ends of the explants or from the abscission layers of the petiole on the node. Since the system was found recalcitrant, it was thought first to develop method for vegetative propagation from highly juvenile material, i.e. from in vitro raised seedlings. Besides the greater plasticity of the juvenile tissues, it is possible to eliminate the problems of disinfecting field grown tissue. After achieving propagation from juvenile material and thus establishing the basic cultural parameters for the species, the method with some modifications was then extended to the clonal propagation of mature trees.

III-1. IDENTIFICATION OF ELITE TREES

To screen tree populations, samples of gum exudate of trees growing wild in the ravines of Mahisagar near Vasad, semiarid areas of Kutch and Sindhroat were collected. Collection of gum-resin was done by tapping, mostly during November to June, as the best exudation is possible only in dry season. Tree exudates are natural products secreted by a tree usually in response to wound injury and include

true gum, resins, gum-resins, oleoresins and mucilages. The exudate of Commiphora wightii is gum-resin, a natural mixture of gum and resin.

The fresh exudate was semisolid which upon exposure solidified in the form of light to dark brown conglomerates of tears, slightly sticky to touch and with a faint balsamic odour. About 5 g of gum exudate was collected from each plant. This material was collected from different areas and carefully preserved in plastic boxes for analysis.

The ketonic fraction, which contains the two important guggulsterones Z and E and also carries almost all the hypocholesterolemic activity was isolated by semicarbazide treatment as described in Materials and Methods (Chapter II-6.a.1). Presence of guggulsterones was confirmed by TLC analysis (Chapter II-6.a.4). Percentage of guggulsterones was determined by UV spectrophotometric method as mentioned in Materials and Methods (Chapter II-6.a.1). The results of this study on gum-resins of three different localities are presented in the Table III-1 (a,b,c).

It is observed from Table III-1 (a,b,c) that the variation in % ketonic fraction in the trees in Vasad ravines was registered from 3.0 to 8.6, whereas % guggulsterones ranged from 1.3 to 3.6. The % ketonic fraction varied from 2.2 to 9.0 and % guggulsterones from 1.0 to 4.0 in trees growing wildly in Kutch area. Screening of eighteen Commiphora

Table III-1.a Screening of Commiphora wightii trees
in Vasad ravines.

Wt. of resin exudate from each plant - 100 mg

Mol. wt. of guggulsterones Z and E - 312

EtOH
max λ 241 nm, ϵ - 27,000

Plant No.	Wt. of EtOAc extractable fraction, mg	Ketonic fraction % of Gum-resin	Guggulsterones % of Gum resin
1	38.2	4.8	2.3
2	40.3	5.4	2.4
3	46.5	8.5*	3.6*
4	36.1	3.9	1.7
5	48.8	8.6*	3.6*
6	33.2	3.0	1.4
7	35.8	3.9	1.8
8	45.7	6.1	2.8
9	37.2	4.5	2.2
10	42.8	5.1	2.4
11	34.2	3.4	1.5
12	32.4	3.1	1.3

* 'elite' trees with higher % ketonic and guggulsterones fraction.

Table III-1. b Screening of Commiphora wightii trees
at Kutch

Wt. of resin exudate from each plant - 100 mg

Mol. wt. of guggulsterones - 312

EtOH λ 241 nm, ϵ - 27,000
max

Plant No.	Wt. of EtOAc extractable fraction, mg	Ketonic fraction % of Gum-resin	Guggulsterones % of Gum-resin
1	35.2	5.8	2.8
2	38.4	5.2	2.3
3	42.5	6.6	3.2
4	48.1	9.0*	4.0*
5	27.8	2.2	1.2
6	34.6	4.5	2.1
7.	28.9	3.1	1.3
8	31.5	3.5	1.4
9	45.1	8.1*	3.8*
10	30.7	4.3	2.0
11	23.2	2.5	1.1
12	32.6	4.3	2.2
13	46.2	8.5*	3.8*
14	22.8	2.5	1.0
15	29.3	3.1	1.4
16	37.4	5.2	2.2
17	28.2	4.8	2.4
18	26.1	3.8	1.9

* 'Elite' trees with higher % ketonic and guggulsterones fraction.

Table III-1.c Screening of Commiphora wightii trees at Sindhroat.

Wt. of resin exudate from each plant - 100 mg
 Mol. wt. of guggulsterones - 312
 EtOH λ 241 nm, ϵ - 27,000
 max

Plant No.	Wt. of EtOAc extractable fraction, mg	Ketonic fraction % of Gum-resin	Guggulsterones % of Gum-resin
1	35.2	3.8	1.8
2	38.8	4.5	2.1
3	40.4	5.6	2.6
4	27.7	3.8	1.9
5	41.2	6.1	2.9
6	45.3	6.7	3.2
7	25.6	2.4	1.2
8	44.6	6.7	3.2
9	38.4	4.9	2.5
10	35.2	4.5	2.4
11	40.6	5.6	2.7
12	30.1	3.6	1.6
13	47.2	8.6*	3.8*
14	28.6	4.2	2.0
15	32.2	3.4	1.4
16	25.8	2.8	1.3
17	28.5	3.1	1.4
18	41.8	6.8	3.3

* 'Elite' plant with higher % ketonic and guggulsterones fraction.

wightii trees from Sindhroat exhibited variation in % ketonic fraction from 2.4 to 8.6 and that of guggulsterones from 1.2 to 3.8.

These Tables clearly indicate genotypic variation with respect to the % ketonic and % guggulsterone fraction in the trees. 'Elite' trees, two from Vasad ravines, three from Kutch and one from Sindhroat were identified with respect to these parameters.

No correlation was however, observed in the ketonic or guggulsterone content with the climatic conditions under which the trees are grown.

The 'elite' trees were used as source of material for further studies.

II-2. IN VIVO PROPAGATION BY STEM CUTTINGS

Stem cuttings of 'elite' trees growing in three different localities had been planted in the University Botanical Garden after giving various treatments. The following treatments were tried :-

1. Cuttings were soaked in water for 18 h,
2. Seradix 1, 2, 3 were applied individually to the cut ends before planting the cuttings,
3. The cuttings were soaked in Seradix 3, 1.0% (w/v) for 18 h before planting.

The success of rooting on the cuttings was found to depend on -

1. size of the cuttings
2. season
3. rooting medium employed.

It was observed that the highest percentage of sprouting as well as survival of the cuttings was obtained with Seradix 3 treatment (both, applied directly and added in water) as presented in Table III.2. The sprouting and survival of cuttings was very poor without any treatment. It was noticed that very thick branches (1 cm diameter) were required for the establishment of cuttings. Smaller branches did not survive even after sprouting. It was further observed that the percentage of sprouting was higher during June to September (Fig. III.1).

Similarly, plantlets were also raised from seeds collected from different regions. The seeds were mechanically scarified, soaked in water for 24 h and planted. The percentage of germination observed to be very low (8 to 10 %). Seed viability and dormancy offer major problems due to hard seed coat.

Table III-2. In vivo propagation by stem cuttings of
Commiphora wightii

Sr. No.	Treatment	Total no. of cuttings planted	No. of cuttings sprouted	% of sprouting	No. of cuttings survived	% of survival
1.	None	112	8	7.1	3	2.6
2.	Water*	100	18	18.0	9	9.0
3.	Seradix 1	56	13	23.2	8	14.2
4.	Seradix 2	52	18	34.6	12	23.0
5.	Seradix 3	58	25	43.1	18	31.0
6.	Seradix + 3 in water	50	21	42.0	15	30.0

* Cuttings soaked in water for 18 h

+ Cuttings soaked in water with Seradix for 18 h

III-3. IN VITRO PROPAGATION FROM SEEDLING MATERIAL

III-3.a. In vitro germination of seeds

The seeds of Commiphora wightii were surface sterilized as described in Materials and Methods (Chapter II-3.a.1). The aseptically dissected embryos were inoculated on hormone free MS half strength medium. Initially the cultures were incubated in dark. Within 4 to 5 days the embryos germinated. After germination the cultures were transferred to light at 1,500 lux, 16 h photoperiod, at $25 \pm 2^\circ\text{C}$. Within 15 days the seedlings attained a height of 6-7 cm. These seedlings served as starting material. (Fig. III.2).

III-3.b. Initiation of cultures and multiplication of shoots

Initially, the selection of auxins and cytokinins and their proportion in the medium for the induction of bud sprouting were done on a trial basis in the light of previous literature available on similar forest trees.

The influence of different cytokinins and auxins alone and in combination on shoot formation from seedling explants was determined using MS medium with 3% sucrose.

In three different sets of experiments Kn, 2-ip and BAP were incorporated in the medium at five different

Fig. III-1 In vivo propagation of Commiphora wightii
by stem cuttings with Seradix 3 treatment.

Fig. III-2 In vitro seed germination of Commiphora
wightii.



Fig. III-1



Fig. III-2

levels ranging from 0.1 to 4.0 mg/l (Table III-3). Zeatin was incorporated at four levels ranging from 0.1 to 2 μ g/l. Different regions of seedling viz. shoot apex, hypocotyl, cotyledonary leaf served as explants. Five replicates were used in each treatment.

The response, percentage of shoot formation and behaviour of the explant (shoot apices) to a particular treatment is recorded in Table III-3; Fig. III-3.

With kinetin alone, only the shoot tip grew within 10 to 15 days and produced a single shoot. The percentage of shoot formation increased with the concentration of kinetin and 40% shoot formation was obtained with 4.0 mg/l kinetin.

The cultures did not response to zeatin or 2-ip either alone or in combination with auxins with respect to the shoot development.

Incorporation of BAP in MS medium improved bud break as well as shoot growth. Kinetin at 4.0 mg/l induced 40% shoot formation, whereas 60% shoot formation was obtained with BAP at 4.0 mg/l.

When only auxins (IAA, IBA, NAA) were incorporated in the medium at different concentrations ranging from 0.1 to 4.0 mg/l, the explants formed only callus at the cut margin without any shoot growth.

Two-three shoots developed with a combination of kinetin and BAP each at 4.0 mg/l within 10-15 days from the shoot apex, showing highest frequency of shoot formation (80%). No callus formation was observed on this medium and shoot development appeared to arise from the axillary buds.

When auxins (IAA, IBA, NAA) were incorporated with cytokinins, callus development accompanied shoot initiation. Moreover, it was also observed that auxins with cytokinins did not improve the shoot induction more than cytokinin alone.

In the initial experiments, activated charcoal was found beneficial for shoot growth. The leaves expanded fully and remained green in presence of activated charcoal. Therefore, activated charcoal ranging from 0.1 to 0.5% was incorporated in the medium.

Green, compact, nodular callus was developed from hypocotyl explants with BAP and NAA at 1.0 mg/l. On other combinations it developed only whitish callus. The other explant viz. cotyledonary leaves, formed only whitish yellow callus on all the combinations tested with no shoot regeneration.

The observations indicated that highest frequency of multiple shoot formation was obtained when two cytokinins

Table III-3

Frequency of response in shoot formation under the influence of various cytokinins and auxins in seedling explants of Commiphora wightii.

Explant - Shoot apex
 Incubation - 30 days at 25±2°C (16 h, 1,500 lux)
 Medium - MS with 3% sucrose

No.	Additives	Response	% shoot formation	Remarks
1.	-	-	-	No response
2.	Kn (0.1)	-	-	No response
	(0.5)	-	-	No response
	(1.0)	+	8	Solitary shoot formation
	(2.0)	+	25	Solitary shoot formation
	(4.0)	+	40	Solitary shoot formation
3.	BAP (0.1)	-	-	No response
	(0.5)	+	20	Single shoot development
	(1.0)	+	38	Single shoot development with slight callusing.
	(2.0)	+	45	Single shoot development with slight callusing at cut end.
4.	(4.0)	+	60	Single shoot development with slight callusing at cut end.
	2-1p (0.1)	-	-	No response
	(0.5)	-	-	" "
	(1.0)	-	-	" "
	(2.0)	-	-	" "
	(4.0)	-	-	" "

Contd...

Table III-3 Contd..

No.	Additives	Response	% shoot formation	Remarks
5.	Zeatin (0.1 μ g l ⁻¹)	-	-	No response
	(0.5 ")	-	-	" "
	(1.0 ")	-	-	" "
	(2.0 ")	-	-	" "
6.	Kn (0.1) + BAP (0.1)	-	-	No response
	Kn (0.5) + BAP (0.5)	+	20	2,3 shoots developed
	Kn (1.0) + BAP (1.0)	+	42	" "
	Kn (2.0) + BAP (2.0)	+	55	" "
	Kn (4.0) + BAP (4.0)	+	80	" "
7.	Kn (4.0) + NAA (0.5)	+	35	Shoot development with callus formation at cut end.
	Kn (4.0) + IAA (0.5)	+	42	Solitary shoot development with callus formation at cut end.
	Kn (4.0) + IBA (0.5)	+	37	Shoot development with callus induction at cut end.
8.	BAP (4.0) + NAA (0.5)	+	55	Solitary shoot development with callusing at cut ends.
	BAP (4.0) + IAA (0.5)	+	58	" "
	BAP (4.0) + IBA (0.5)	+	62	" "

Five replicates were used for each treatment.

Auxins NAA, IBA and IAA when incorporated without cytokinins induced only callus formation without shoot development at the cut margins at all the combinations tested.

Fig. III-3 Response of shoot tips (seedlings) to cytokinins :

A. Kn (4.0 mg l^{-1})

B. BAP (4.0 mg l^{-1})

C. Kn (4.0 mg l^{-1}) + BAP (4.0 mg l^{-1})

(X 2.0)

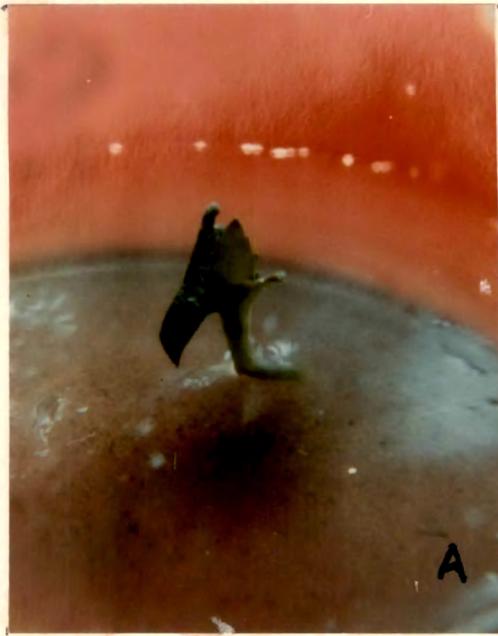


Fig III-3

Kn and BAP were combined together at higher concentration i.e. 4.0 mg/l of each.

III-3.c. Growth of shoot cultures in vitro

In the previous experiment, it was shown that upto 80% multiple shoot formation could be achieved on MS basal medium containing 3% sucrose and supplemented with Kn and BAP each at 4.0 mg/l and activated charcoal (0.1%). It is interesting to note that the shoot apices formed multiple shoots only when the two cytokinins Kn and BAP were combined thus indicating a synergistic effect of cytokinins. The frequency of response also rose from 8 to 40% for Kn, 20 to 60% for BAP, to 20 to 80% with Kn+BAP.

However, the shoots did not elongate in the same medium. Slowly the leaves turned yellow, dropped and explants died if they were maintained on the same medium for more than 30 days. A number of experiments were conducted with glutamine, adenine and phloroglucinol to avoid the premature leaf drop.

III-3.c.1. Effect of Glutamine, Adenine and Phloroglucinol on shoot development

To study the effect of glutamine on shoot development, glutamine at 50, 100, 200 and 400 mg/l was incorporated into MS medium supplemented with 4.0 mg/l Kn and BAP, 3% sucrose and activated charcoal 0.1%.

Shoot apices were cultured on this medium. Incorporation of glutamine was found beneficial for the growth of shoot cultures. The shoots did not exhibit a pronounced elongation, but the premature leaf drop was controlled with glutamine at 100 mg/l. Further increase in the concentration of glutamine did not show any pronounced effect.

Adenine was incorporated in the medium at four different levels, 10, 20, 40 and 80 mg/l. The effect of phloroglucinol (PG) was tested at three different levels, $0.5 \times 10^{-3}M$ or 81 mg/l, $1.0 \times 10^{-3}M$ or 162 mg/l and $2.0 \times 10^{-3}M$ or 324 mg/l. No positive response was observed with phloroglucinol, moreover, it was found to be toxic at higher level i.e. 324 mg/l. Adenine also did not improve leaf retention at any of the concentrations tested.

III-3.c. 11. Effect of varying levels of thiamine HCl on growth and multiplication

There are some reports that thiamine HCl plays an important role in the development of shoots in forest and fruit trees. Hence the levels of thiamine HCl in the medium were also tested. To study the effect of varying levels of thiamine HCl on growth 0.5, 2.0, 5.0 and 10.0 mg/l of thiamine HCl was incorporated into MS basal medium supplemented with Kn and BAP 4.0 mg/l each, 3% sucrose and

activated charcoal 0.1%. No apparent change in the behaviour of the culture was observed in the first three to four weeks in presence of various levels of thiamine in the culture. After 4th week, however, the cultures grown in presence of higher levels of thiamine (5 and 10 mg/l) were much healthier and continued to grow even after 40 days. Cultures grown on MS medium supplemented with Kn and BAP 4.0 mg/l of each, 3% sucrose and 0.1% activated charcoal with normal level (0.1 mg l^{-1}) of thiamine served as control.

From the above results MS medium supplemented with 4.0 mg/l of Kn and BAP, 3% sucrose, 100 mg/l glutamine and 10.0 mg/l thiamine HCl was used as maintenance medium for the shoot cultures.

It was observed that even though the shoots grew well with glutamine and increased levels of thiamine, the shoots exhibited no pronounced elongation.

III-3.d. Elongation of shoot cultures

To stimulate elongation of shoots and to obtain a favourable leaf morphology, three factors, gibberellic acid, state of medium and activated charcoal, reduced levels of cytokinins were tested.

III-3.d. i. Effect of GA₃ on elongation of shoots

GA₃ at five different levels ranging from 0.1 to 4.0 mg/l (0.1, 0.5, 1.0, 2.0, 4.0) was incorporated into MS basal medium containing 4.0 mg/l each of Kn and BAP, 10.0 mg/l thiamine HCl and 100.0 mg/l glutamine. The shoots grown on maintenance medium were carefully excised and inoculated on the above media. Cultures grown on maintenance medium served as control. Shoots incubated at the lower concentration (0.1, 0.5 mg/l) of GA₃ did not show any beneficial effect, whereas the leaf formation in media containing higher concentrations of GA₃ (2.0 and 4.0 mg/l) was severely suppressed. The cultures also became very weak at these levels of GA₃.

III-3.d. ii. Effect of state of medium and activated charcoal

In the previous studies a semi-solid agar medium was used. In this set of experiments the following treatments were compared for the elongation of shoots :

1. Transfer of shoots to fresh liquid medium,
 - a. 20 ml/tube with filter paper support. In this experiment only the lower 3-5 mm of the explant was dipped in the medium.

- b. Shaken in an Earlenmeyer flask on rotary shaker at 100 r.p.m.
 - c. Stationary flask cultures with explants submerged in flasks.
2. Addition of activated charcoal (0.3%) to the liquid medium in tubes.
 3. Addition of 20 ml of liquid medium with charcoal (0.3%) to the established culture

Cultures transferred to the freshly made semi-solid medium of the same composition served as control.

The shoots growing on maintenance medium were carefully excised and transferred to the different treatments.

In control, the shoots multiplied but did not elongate. In liquid medium either with the explants submerged or rotated, no growth occurred and the explants turned brown, while in stationary liquid culture in tubes supported with filter paper, the shoots elongated and grew in the first two weeks after which they turned hard and brittle and died.

Treatments 2 and 3 prevented the hardening of shoots to some extent suggesting beneficial effect of charcoal, but the elongation of shoots was not satisfactory. Perhaps this may be due to the after-effect/carry-over of high BAP

and Kn (4.0 mg/l) concentration in the medium, which is optimal for the multiple shoot formation. (Fig. III-4).

III-3.d. iii. Effect of reduced levels of Kn and BAP on shoot development

The shoots grown on the maintenance medium were carefully excised and transferred to MS medium with reduced levels of Kn and BAP (0.4 mg/l), glutamine 100 mg/l, and thiamine HCl 10.0 mg/l, as under :-

1. Liquid medium with filter paper support.
2. Solid medium of same composition with activated charcoal (0.5%).

It was observed that, the shoots elongated in the liquid medium with reduced concentration of cytokinins. However, at the cut end, callus formation accompanied shoot development.

In solid medium with activated charcoal, shoots elongated and attained a height of 2 to 2.5 cm within 2 to 3 weeks with well expanded leaves (Fig. III-5).

III-3.e. Studies on induction of roots in shoot cultures of *Commiphora wightii*

This part of the section describes the experiments conducted to induce rooting of excised in vitro raised shoots obtained from aseptic seedlings of *Commiphora wightii*.

**Fig. III-4 Effect of state of medium and AC on
shoot elongation :**

- A. liquid medium with filter paper support,**
- B. liquid medium with AC (0.3%),**
- C. addition of 20 ml liquid medium with
AC (0.3%) to the established culture.
Note that the shoots remained green
with AC.**

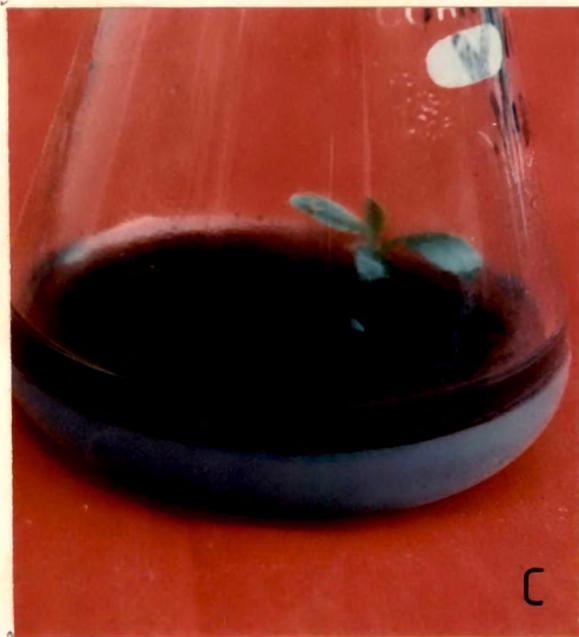
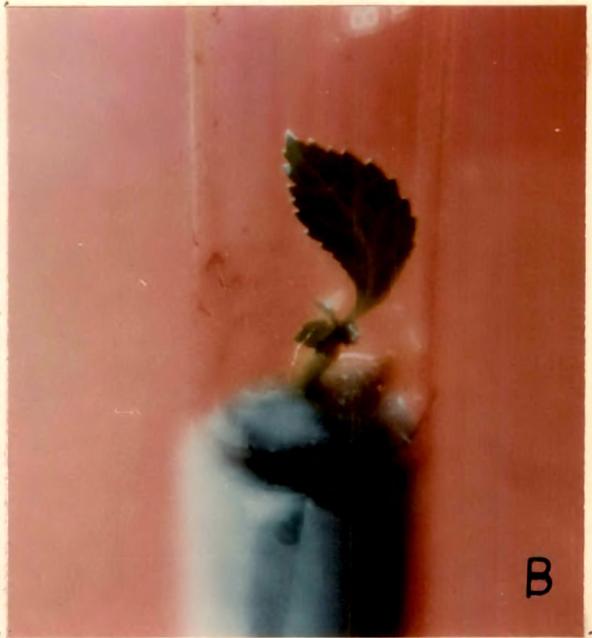


Fig. III-4

The experiments were conducted both for primary and subcultured shoots which had undergone two subcultures. All experiments were conducted in replicates of three, the shoots in rooting media were incubated at $25 \pm 2^\circ\text{C}$ with 1500 lux for 16 h unless otherwise stated.

III-3.e. 1. Effect of auxins

Different auxins viz. IAA, IBA and NAA which are known to initiate and promote root formation were tested at different concentrations (Table III.4). IAA, IBA and NAA were separately incorporated into half strength MS and White's media containing 3% sucrose. The media were poured in test tubes with filter paper support and contained auxins at the concentrations 0.1, 1.0 and 5.0 mg/l. None of the auxins added singly at the concentration tested induced root formation. Various combinations thereof were tested at different concentrations. Roots were induced with the combination of IAA and IBA at 1.0 and 5.0 mg/l respectively. However, prolonged auxin treatment caused callus formation. Even the newly initiated roots started callusing because of prolonged treatment with auxins. Moreover, the growth of the shoots was also affected.

Experiments were, therefore, conducted to overcome the inhibitory effect of auxins by short term exposure of shoots to root inducing medium (RIM).

Table III-4 Effect of auxin combinations on rhizogenesis.

Medium : Half strength MS medium with 3% sucrose.

Supplements : Auxin combinations and concentrations tested.

Auxins	Concentrations in mg l ⁻¹		
	0.1	1.0	5.0
IAA + IBA	-	+	+
IAA + NAA	-	-	-
IBA + NAA	-	-	-

- No root initiation

+ Root initiation

III-3.e. ii. Effect of duration of exposure to root inducing medium (RIM)

Individual shoots were exposed to RIM for different time intervals in dark and then the cultures were transferred to rooting medium (RM) which is hormone free half strength MS or White's medium with 3% sucrose. Cultures not transferred to RM and cultures transferred to RM without exposing to RIM served as control. IAA and IBA were added at 1.0 mg/l in RIM.

Rooting was induced in two treatments i.e. after 24 and 48 h treatment in dark with a combination of two auxins IAA and IBA at 1.0 mg/l (Table III-5).

A 12 h treatment with these auxins in combination was insufficient, whereas treatment for prolonged periods; larger than 48 h, caused callusing at the base of the shoots.

Initiation of roots was observed within 7 to 8 days after transfer to RM.

Highest frequency of rooting 60% was obtained when shoots were transferred to half strength MS medium with activated charcoal (0.3%) (Fig. III.6). 25% shoots rooted on half strength MS medium, while only 4% shoots rooted on White's medium.

Table III- 5 Effect of period of treatment with the combination IAA + IBA (1 mg l^{-1})

Auxin treatment was given in dark at 25°C .

Period of treatment (h)	Medium	Root initiation	% Root initiation	Callus formation
12	MS 1/2	-	-	-
24	MS 1/2	+	25	-
48	MS 1/2	+	20	+
12	MS 1/2 + activated charcoal (0.3%)	-	-	-
24	"	+	60	-
48	"	+	40	+
12	White's	-	-	-
24	"	+	4	-
48	"	+	4	+

- No root initiation, No callus formation

+ Root initiation, Slight callus formation

Fig. III-5 Shoot elongation with reduced levels of Kn and BAP (0.4 mg l^{-1}), glutamine (100 mg l^{-1}), thiamine HCl (10 mg l^{-1}) and AC (0.5%).

Fig. III-6 Induction of roots in MS 1/2 + AC (0.3%) after 24 h pretreatment in dark with a combination of IAA and IBA (1.0 mg l^{-1}).



Fig.III-5



Fig.III-6

Cultures transferred to RM without exposing to RIM did not form any roots. Cultures in continuous contact with RIM formed roots within 8-10 days; but callusing accompanied root formation and the presence of auxins inhibited further growth of shoot system.

III-4 CLONAL PROPAGATION OF ELITE CLONES

In light of the results obtained in the experiments conducted with in vitro raised seedlings, experiments were designed for the adult material. Three elite clones (two from Vasad and one from Sindhroat), identified on the basis of chemical investigation, were selected for further experiments. Experiments were not conducted with the three elite clones identified in Kutch area because of practical difficulty.

III-4.a Seasonal effect

It is now well known that the season in which buds are cultured has a profound effect on their response in culture. A strong seasonal effect is observed in this particular tree. Vegetative buds are available on the tree only from April till October. After October defoliation starts and the trees are without any buds or leaves from November till March. In this experiment, explants collected in different months from April to October were cultured on MS medium supplemented

with 3% sucrose, 4.0 mg l⁻¹ each of Kn and BAP, glutamine 100 mg l⁻¹, thiamine HCl 10 mg l⁻¹ and activated charcoal 0.1% (MS-1). Plant material collected in the months of April-June gave good response with 30 to 40% of the buds sprouting, whereas explants collected between July-August, the percentage of sprouting was reduced 10 to 20%. Moreover, the contamination rate was higher in spite of giving nystatin treatment. During ~~Sept~~ Sept-October period, the percentage of sprouting was poor 5%. This seasonal effect was, however, not observed in the subculture (Fig. III-7).

Moreover, since the material was collected from forest, it offered serious problems for getting sterile cultures. Endogenous microbial contaminations were often present resulting in high contamination rates. Removal of these contaminants was found very difficult. After making numerous attempts with antibacterial and antifungal treatments, it was possible to obtain 90% sterile cultures by treatment with nystatin and streptomycin as described in chapter II-3.a.2.

III-4.b Source of explant

Different explants such as terminal bud (5-8 mm), isolated axillary buds (1-2 mm), nodal segments (10-15 mm) with axillary buds were inoculated on MS-1 medium, and incubated at 25±2°C, at 16 h photoperiod with 1,500 lux.

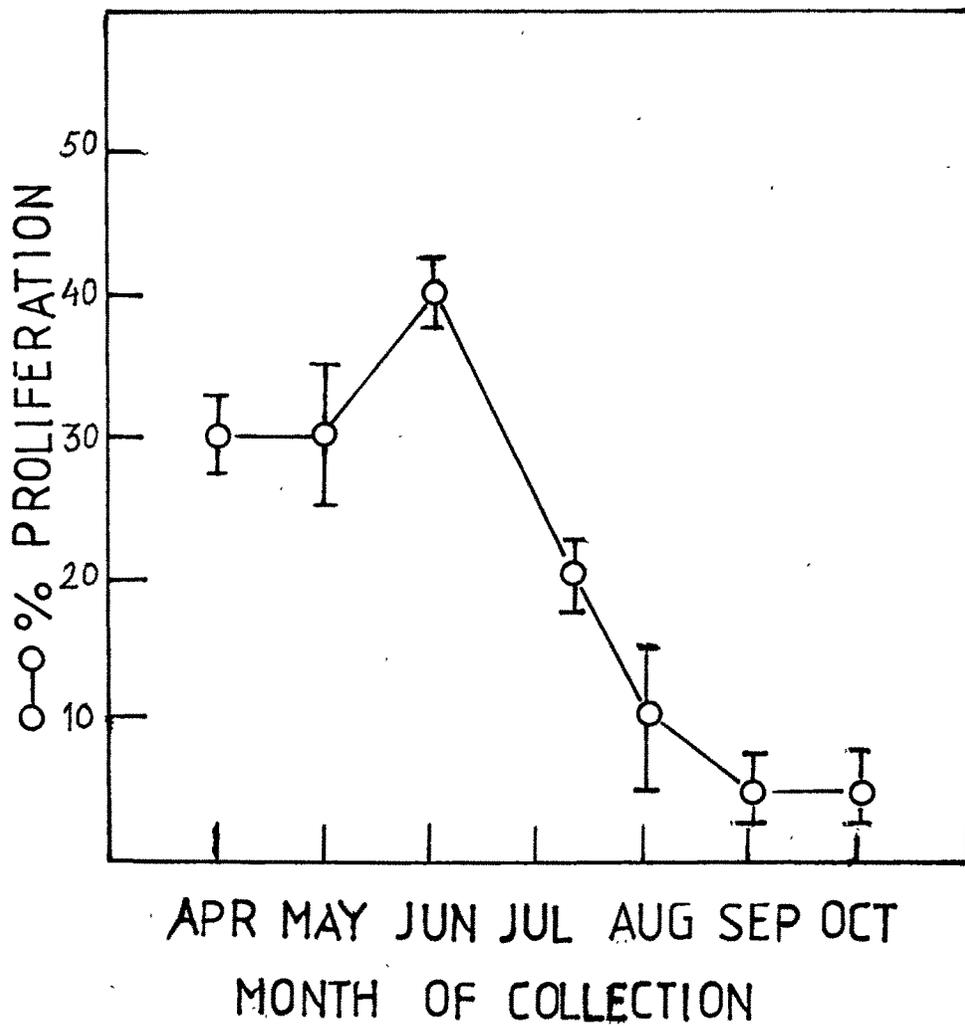


Fig. III-7 Influence of season on shoot proliferation from the explants derived from mature Commiphora wightii trees.

Only nodal segments with axillary buds gave rise to shoots, while the terminal and single axillary buds turned brown and died. As a result of this experiment only nodal explants were used for further studies.

III-4.c Frequency of response in the induction of bud burst in *Commiphora wightii*

Even though, the nodal explants responded in the culture, the frequency was observed to be low (30-40%). So an experiment was designed to increase the frequency of bud burst in culture. Shoot tips were excised from the actively growing shoots of mature trees and the successive nodal explants with dormant axillary buds were cultured on various days after excision of the terminal bud. The results are summarised in Table III-6. It was observed that the first nodal explant (axillary bud 1.0 to 1.5 mm, unopened) exhibited 85% induction of bud burst when cultured on 8th day after excision of the terminal bud.

Therefore, these nodal explants were preferred as inoculi for subsequent experiments.

III-4.d Influence of cytokinins, activated charcoal, folic acid and biotin in the induction of bud burst

Results obtained with the seedlings explants indicated that shoot formation was obtained only with the cytokinins,

Table III-6 In vitro response for the induction of bud-burst in C. wightii explants

Nodal explant with axillary bud cultured after excision of terminal bud

Days	Size of bud	No. of buds cultured	No. of buds sprouted	% of sprouting
2nd	not visible	20	0	0
4th	1 mm (unopened)	20	0	0
6th	1 mm (unopened)	20	7	35
8th	1-1.5 mm (unopened)	20	16	80
10th	1.5-2 mm (some buds are opened)	20	9	45

Different nodal explants cultured on 8th day

Nodes	Size of bud	No. of buds cultured	No. of buds sprouted	% of sprouting
1st	1-1.5 mm (unopened)	20	17	85
2nd	1.0 mm (unopened)	20	11	55
3rd	0.05-1.0 mm	20	06	30
4th	not visible	20	00	00
5th	not visible	20	00	00

Kn and BAP. Multiple shoots were initiated on a media containing both these cytokinins. The other two cytokinins zeatin and 2-ip were not found effective. Moreover, it was also observed that auxins tend to initiate callus formation at the cut ends. So they were not tried for adult material.

To study the influence of bud burst Kn and BAP were added separately in MS medium with 3% sucrose supplemented with 100 mg l^{-1} glutamine and 10 mg l^{-1} thiamine HCl, at the concentration ranging from 0.1 to 4.0 mg l^{-1} . All the media contained activated charcoal 0.1%. In another set of experiments Kn and BAP were incorporated in the medium at different concentrations (0.1 to 4.0 mg l^{-1}).

The nodal explants with dormant axillary buds were cultured on the media. It was observed that with Kn alone at higher concentration 4.0 mg l^{-1} , 60% buds sprouted thus producing a solitary shoot. About 80% buds sprouted when BAP was incorporated in the medium at higher concentration (4.0 mg l^{-1}). Solitary shoots were developed with BAP also but the shoots were healthier than produced with Kn alone. Multiple shoots (2 to 3 shoots per explant) were obtained in 80% of the cultures when Kn and BAP were incorporated in the medium at higher concentration 4.0 mg l^{-1} of each (Fig. VII-8).

Fig. III-8 Response of nodal explants (mature trees)

to cytokinins :

A. Kn (4.0 mg l^{-1})

B. BAP (4.0 mg l^{-1})

C. Kn (4.0 mg l^{-1}) + BAP (4.0 mg l^{-1})

(X 1.4).



Fig. II-8

However, it was observed that addition of glutamine (100 mg l^{-1}), thiamine HCl (10.0 mg l^{-1}) and activated charcoal (0.1%) in the medium were not sufficient to prevent the premature leaf drop. It was further observed that by increasing the level of activated charcoal in the medium (0.5%), the cultures survived and remained green for longer time (Fig. III-9). Incorporation of Folic acid (0.1 mg l^{-1}) and Biotin (0.1 mg l^{-1}) was also found beneficial for the growth of the cultures and helped the cultures to survive for a longer time. So for the maintenance medium of adult material Biotin and Folic acid were routinely incorporated in the medium at 0.1 mg l^{-1} . Various concentrations of Folic acid and Biotin were tried but they did not exhibit any significant change except the one already mentioned.

III-4.e Elongation of shoot cultures

In light of the results obtained with the juvenile material, an experiment was designed for the elongation of shoots obtained from adult material. The shoots were excised and transferred to

1. solid medium of the same composition,
2. liquid medium of the same composition,
3. liquid medium with activated charcoal (0.3%),
4. liquid medium with reduced levels of Kn and BAP 0.4 mg l^{-1} of each.

Fig. III-9 Effect of AC (0.5%) on shoot development.
Note that increased level of AC
prevented premature leaf drop.



Fig. III-9

5. solid medium with reduced levels of Kn and BAP
0.4 mg l⁻¹ of each with activated charcoal 0.5%.

All these media contained glutamine 100 mg l⁻¹, thiamine HCl 10.0 mg l⁻¹, Biotin and Folic acid 0.1 mg l⁻¹ of each.

In solid medium with higher levels of Kn and BAP, the shoots multiplied but did not elongate. So this medium was used for the subculture of shoot cultures (MS-2). When the shoots were transferred to liquid medium of the same composition, it turned rigid, vitrified and the leaves turned yellow. A beneficial effect of activated charcoal was also observed in mature cultures. The shoots remained green and viable for more than two months without showing any elongation. The shoots elongated to some extent in liquid medium with reduced levels of Kn and BAP (0.4 mg l⁻¹ of each), but in liquid medium, the leaves turned yellow. In solid medium with reduced levels of Kn and BAP, and activated charcoal 0.5%, the shoots elongated and attained a height of 2 to 3 cm. within 15 days. (Fig. III-10).

It was further observed that glutamine 100 mg l⁻¹ which was required in the maintenance medium was not required for the further growth. So it was eliminated from the elongation medium.

Hence MS medium with 3% sucrose supplemented with Kn and BAP at 0.4 mg l⁻¹, thiamine HCl 10.0 mg l⁻¹, Folic acid

Fig. III-10 Effect of state of medium and AC on shoot elongation (explants from mature trees) :

- A. liquid medium with Kn and BAP (4.0 mg l^{-1}) and AC (0.3%)
- B. liquid medium with reduced levels of Kn and BAP (0.4 mg l^{-1})
- C. solid medium with AC (0.5%) and reduced levels of Kn and BAP (0.4 mg l^{-1}).



Fig.III-10

and Biotin 0.1 mg l^{-1} and activated charcoal 0.5% was used as elongation medium for mature explants (MS-3).

III-4.f Induction of roots in shoot cultures
(mature explants)

As indicated in the results obtained with seedling explants that treatment of the auxins IAA, IBA and NAA singly to the shoot cultures failed to produce roots. So these treatments were not tried for the mature explants.

In one set of experiment the excised shoots were treated with a combination of IAA and IBA at different concentrations 0.1, 1.0 and 5.0 of each and after different period of treatment (12, 24, 48 h) transferred to half strength of MS medium.

In another set of experiment the shoot explants were treated with a combination of IAA and IBA as above and transferred to half strength MS medium with activated charcoal 0.5%.

It was observed that in both the cases the shoots rooted when they were treated with a combination of IAA and IBA at 1.0 mg l^{-1} and 5.0 mg l^{-1} of each for 24 and 48 h. However, an improved root system with root laterals was obtained when charcoal 0.5% was incorporated in the rooting medium (Fig. III-41).

Fig. III-11 Induction of roots in shoot cultures
(mature explants) in MS 1/2 + AC (0.5%)
after pretreatment with IAA + IBA (1.0 mg l^{-1})
for 24 h in dark.



Fig. III-11

III-4.g Elongation of shoots after rooting

It was observed that the rooted plantlets did not elongate further in the rooting medium. The growth remained stunted. A number of experiments were conducted for further elongation. Addition of Kn, BAP at reduced levels, addition of GA₃ were tried but without much success.

So it was then thought of trying different salt concentrations with reduced levels of Kn and BAP. MS, Gamborg's, Wood and Broun's, White's mineral salt compositions were tried at full and half strength with vitamins at full strength of MS.

It was observed that on half strength of Wood and Broun's medium (1961) the shoots elongated and attained a height of 5 to 6 cm within 10 to 15 days without inhibiting the root system. The internodal length increased. Sturdy plantlets with well developed root system were obtained (Fig. III-12).

III-4.h Transfer to soil

When the rooted plantlets on half strength of Wood and Broun's medium had attained a height of 50 to 60 mm, they were transferred to earthen pots containing a sterile vermiculate:sand (1:1) mixture. They were watered daily with tap water and twice per week with one fourth strength MS

Fig. III-12 Elongation of shoots after rooting

A. in 1/2 MS medium

B. in 1/2 WB medium.



Fig. III-12

mineral salts for the first 10 days and then with tap water. The plantlets were covered with inverted glass beakers to maintain humidity and kept in culture room with 16 h photoperiod (1,500 lux) at $25 \pm 2^\circ\text{C}$. The plantlets were acclimatized by the removal of the beaker, few hrs in the beginning and then completely after 2 weeks. With the onset of new leaves within 8 days, they were transferred to the Green house (Fig. III-13).

III-5 STUDIES ON MORPHOGENESIS IN CALLUS CULTURES

As the callus obtained from hypocotyl explants was green and compact in nature (Fig. III-14) in contrast to the whitish, yellow, friable callus obtained on MS medium with Kn and 2,4-D (Chapter IV), attempts were made to differentiate this callus to produce plantlets.

III-5.a Effect of varying the strength of nutrient medium on morphogenesis

To induce shoot buds in a culture derived from hypocotyl and maintained on MS medium containing 3% sucrose and supplemented with 1 mg l^{-1} ^{BAP} and NAA a number combinations were tried. The callus was transferred to different strengths of MS basal medium (one fourth, one half and full strength). But these treatments failed to invoke any morphogenetic response. When the callus was transferred to basal media of

Fig. III-13 Commiphora wightii plantlet transferred
to pot (2 months old).



Fig. III-13

all strengths, it lost its green appearance to some extent and it became whitish, pale-green, compact callus. Whitish filamentous protruberances were seen all over the cultures (Fig. III-15). None of the treatments favoured organogenesis or embryogenesis.

III-5. b Effect of auxins and cytokinins individually and in combination on morphogenesis

To examine the hormonal influence individually and in combination on morphogenesis if any, three auxins and two cytokinins were tested individually and in combination. Callus cultures maintained on MS medium supplemented with BAP and NAA at 1.0 mg l^{-1} and 3% sucrose (on which the callus has undergone two subcultures) was transferred to experimental media containing hormones singly or in combination as shown in Table III-7.

Cultures were observed periodically for any morphogenetic response. The results obtained at the end of incubation period of 35 days are presented in Table III-7.

At higher concentrations of IAA and IBA 5.0 mg l^{-1} , roots were induced from the callus in the 2nd week of culture. The effect was more pronounced when activated charcoal (0.5%) was incorporated in the medium. With NAA also at higher concentration (5.0 mg l^{-1}) root induction

Table III-7 Effect of auxins and cytokinins on morphogenetic response in callus cultures.

Inoculum : Callus cultures induced from hypocotyl grown on MS medium with BAP and NAA at 1 mg l^{-1} .

Medium : MS medium with 2% sucrose. The auxins and cytokinins were added as indicated below.

Incubation: 30 days at $25 \pm 2^\circ \text{C}$ (1,500 lux, 16 h photoperiod).

Auxins	mg l^{-1}	Cytokinins	mg l^{-1}	Morphogenetic response
1. IAA	0.2	-	-	-
	1.0	-	-	-
	5.0	-	-	R +
2. IBA	0.2	-	-	-
	1.0	-	-	-
	5.0	-	-	R +
3. NAA	0.2	-	-	-
	1.0	-	-	-
	5.0	-	-	R ++
4.	-	Kn	0.2	-
			1.0	-
			5.0	-
5.		BAP	0.2	-
			1.0	-
			5.0	-
6. IAA	0.2	Kn	5.0	-
	5.0		0.2	-
7. IBA	0.2	Kn	5.0	-
	5.0		0.2	-
8. NAA	0.2	Kn	5.0	-
	5.0		0.2	R ++
9. IAA	0.2	BAP	5.0	-
	5.0		0.2	R +
10. IBA	0.2	BAP	5.0	-
	5.0		0.2	R +
11. NAA	0.2	BAP	5.0	-
	5.0		0.2	R ++

R + , 10 to 40% of cultures

R ++, 40 to 60% of cultures

was obtained. Moreover, number of cultures forming roots was more with NAA as compared with IAA or IBA. Kn and BAP alone at low concentration supported callus growth and the callus grew as green, compact callus without any morphogenetic response. At higher level of both the cytokinins, the growth was comparatively reduced.

The root induction was further enhanced at high concentration of NAA 5.0 mg l^{-1} and low level of BAP 0.2 mg l^{-1} (Fig. III-16). When the auxin-cytokinin levels were reversed (i.e. high cytokinin to low auxin) to induce shoot formation, the root formation was inhibited without any shoot induction.

The cultures responded more effectively to the auxin NAA and cytokinin BAP than other two auxins i.e. IAA and IBA and cytokinin Kn. But a wide range of trial treatments including those not recorded here, failed to invoke any regeneration of shoots.

Fig. III-14 Green, nodular callus developed from hypocotyl in MS + BAP and NAA (1.0 mg l^{-1}).

Fig. III-15 Callus transferred to basal medium.

Fig. III-16 Rhizogenesis with NAA (5.0 mg l^{-1}) and BAP (0.2 mg l^{-1}).



Fig.III-14

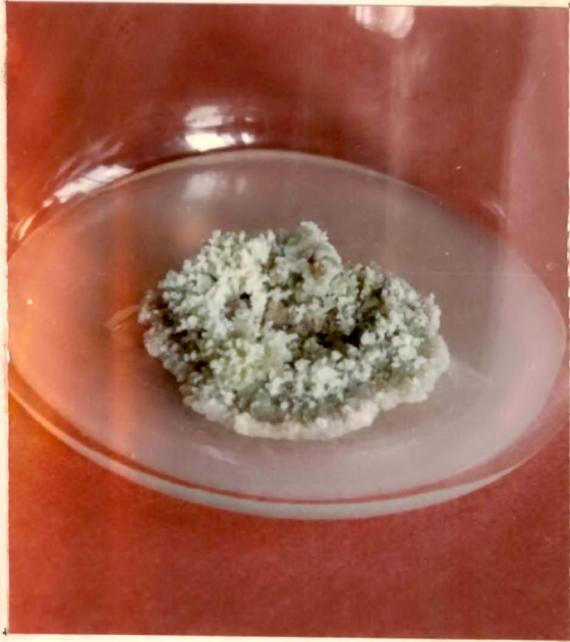


Fig.III-15



Fig.III -16