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Clonal propagation studies of Alectra chitrakutensis and Butea superba critically endangered and rare medicinal plants

Alectra chitrakutensis (Kanda)

Butea superba (Belapalash)

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Chapter-1

Introduction

Conservation and propagation of medicinal plants is also one of the major key components of research. Generally, medicinal plant are conserved and propagated through *in situ* and *ex situ* methods. However, these techniques requires huge space and labour cost. Most of the medicinal plants are generally propagated by seeds, whole plants, bulbs, tubers or through cuttings etc. In case of RET species there are limitations to propagate them by conventional propagation methods such as seeds, whole plant, bulb etc. due to non availability of appropriate amount of propagation material. So, biotechnological interventions are now available through which RET species can easily be multiplied and conserved. Macropropagation and Micropropagation are the best biotechnological applications through which small amount of propagation material can easily be propagated and multiplied successfully. Several workers reported that rare critically endangered and threat species can easily be multiplied by using tissue culture technique (Ahuja *et.al.* 1987, Mathur *et.al.* 1990, Kukraja *et.al.* 1990, Tiwari *et.al.* 2000, 2002).

In India several research and development laboratories are actively engaged in multiplying such RET species. Propagation of plant through macropropagation is also one of the most useful technique for the multiplication of such plant species. For this, mist chambers or polypropagators are normally used for such purpose. This is an easy and cheaper technique as compared to tissue culture technique. *Alectra chitrakutensis* (Rau) Prasad and Dixit is commonly known as Kanda, Medkikand belongs to family Serophulariaceae is a critically endangered annual herb includes more than 50 species of parasitic herbs distributed in India, Africa, South America and Australia. A majority of the species is found in tropical Africa and some are common to Australia. Two species are recorded from Brazil, one from Arabia and three from Madagascar. The three members from India are (1) *Alectra indica*. Benth (2) *A. thomsonii* Hook.f. and (3) *A. parasitica* A.Rich var.chitrakutensis (Rau) R.Prasad and R.D. Dixit. Vegetatively it can be distinguished by strongly rhizomatous development of the underground part of the stem. The perennial whole plant strands up shoots from the numerous buds in the axils of closely set scale leaves.

Madhya Pradesh is the most suitable for the growth and development of this species particularly in the natural vicinity of Chitrakoot along the borders of Satna (M.P.) and Banda District of Uttar Pradesh. For Madhya Pradesh it is reported to be critically endangered medicinal plant (http://envis.frlht.org/index.php/bot_search). It is always found growing in a parasite on the roots of *Vitex negundo* Linn which is called midaki in Chitrakut region. *Alectra chitrakutensis* is traditionally used for the treatment of leprosy for centuries in traditional Ayurveda (Bedi, 1967). It is also used in the treatment of various ailments such as tuberculosis, paralysis, piles, intestinal worms, constipation, leucorrhoea, fever, spermatorrhoea and also as blood purifier. Due to its high economic value and used in pharmaceutical industries the natural population of this species is declining rapidly due to which this genus is at the verge of extinct. During the survey under this study it was noticed that this species is only available in few pockets in Chitrakoot region. Therefore, it is an urgent need to domesticate, protect, conserve and multiply this species immediately.

Butea superba (ROXB) is commonly known as Belapalash belongs to family Fabaceae. It is climber in nature. It is mainly found in Malaysia, Indonesia, Vietnam, Sri Lanka and India. In India it is found in Uttar Pradesh to West Bengal, Madhya Pradesh, Chhattisgarh, some part of Maharashtra, Assam and southwards to Peninsular India (http://envis.frlht.org/index.php/bot_ search). In Madhya Pradesh, it is distributed throughout in the state. However, due to over exploitation and unsustainable harvesting practices of this species since last few decades its natural population from the states is declined rapidly, due to this the species comes under near threat (NT) category. (http://envis.frlht.org/index.php/bot_search).

The roots of Butea superba are used as a source of medicine. Traditionally people take it for sexual performance problems and lack of interest in sexual activity. The roots are also used in treatment of diarrhea, painful difficult urination and fever.

Due to their high medicinal value the natural population of both the medicinal plant species are declining rapidly. Therefore, today it is an urgent need to domesticate, conserve and multiply clonely. In this technical bulletin the clonal propagation technique have been described for the multiplication of *Alectra chitrakutensis* and *Butea superba*.

Chapter-2

Objectives

1. To collect wild germplasm of designated species from different forest area of Madhya Pradesh.
2. To standardized and multiply the identified germplasm by standardizing clonal propagation protocol.

Chapter-3

Material and Methods

As per the available literature regarding their availability in Madhya Pradesh reconnaissance survey was made in different forest areas of M.P. for the collection of plant material in the form of whole plant, cuttings and roots for further studies. The northern part of the state particularly Chitrakoot forest area was found the hot spot of *Alectra chitrakutensis* while *Butea superba* was found almost in entire state.

a. Method for clonal macropropagation of *Alectra chitrakutensis*

Alectra chitrakutensis is a tiny parasitic plant grows alongwith the roots of Nirgundi (*Vitex negundo* L). Its macropropagation is not possible through its rhizomatic tuber. However, an attempt have been made to propagate it by rhizomatic tubers without any hormonal treatment. The rhizomatic tubers were segregated from host plant and were directly shifted in the open beds with the rooted cuttings of host plants during November-December. The new plants *Alectra chitrakutensis* were emerges during March to April.

Rhizomatic tubers of *Alectra chitrakutensis*

b. Standardization of micropropagation protocol : An attempt has been made for standardizing micrpropagation protocol of *Alectra chitrakutensis* and *Butea superba* from nodal ex-plant, apical parts and tubers respectively.

Steps involved in micropropagation : The most important aspect of the *in vitro* techniques is to carry out various operations under aseptic conditions. In order to

maintain the aseptic environment inside the culture vials, the following necessary measures were taken for the standardization of micropropagation protocol.

(1) Selection of appropriate explants for *in vitro* multiplication.

(2) Standardization of proper sterilization techniques of explants for aseptic culture.

(3) Standardization of *in vitro* protocol on culture medium (Murashige and Skoog's Medium 1962) with various combination and concentration of plant growth regulators.

(4) Initiation of shoot regeneration.

(5) Growth and multiplication of shoots.

(6) Induction of roots in regenerated shoots.

(7) Hardening and acclimatization of tissue cultured plants.

Culture Media used for tissue culture

Composition of modified Murashige and Skoog's (MS 1962) Medium:

Compound	Basal Medium Amount (mg/l) (1962)	Stock Solutions Amount (mg/l)
Stock I (macronutrients)		
NH_4NO_3	1650	33000
KNO_3	1900	38000
$MgSO_4.7H_2O$	370	7400
$CaCl_2.2H_2O$	440	8800
KH_2PO_4	170	3400
Stock-II (micronutrients)		
KI	0.83	166
H_3BO_3	6.2	1240
$MnSO_4.4H_2O$	22.3	4460
$ZnSO_4.7H_2O$	8.6	1720
$NaMoO_4.2H_2O$	0.25	50
$CuSO_4.5H_2O$	0.025	5
$CoCl_2.6H_2O$	0.025	5
Stock III (iron stock)		
$FeSO_4.7H_2O$	27.8	5560
$Na_2EDTA.2H_2O$	37.3	7460
Stock IV (vitamins)		
Inositol	100	20000
Nicotinic acid	0.5	100

Table cont...

Pyridoxine HCl	0.5	100
Thiamine HCl	0.1	20
Glycine	2	400
PGR	- as per need	
Sucrose	- 30% (30 gm/l)	
pH	- 507 – 508 using 1N HCl or 1N Na OH	
Agar	- 0.8% (8 gm/l)	

Preparation of stock solutions:

The methodology for media preparation involves preparation of stock solutions (in the range of 10 to 100% conc.) of highly purified chemicals and high grade double distilled water. The stock solutions were stored in glass or plastic containers or frozen till further requirements.

1. Each component was dissolved separately to the last particle and then mixed with the others.
2. All the stock solutions were stored in proper plastic or glass bottles in refrigerator.
3. The iron stock was stored in an amber colour bottle.
4. Glaswares, double distilled water and chemicals were used of high purity for preparing stock solutions and media.

Stocks amount used in the media

Volume of Stock Solutions	Volume of media			
	10 Ltr.	4 Ltr.	2 Ltr.	1 Ltr.
Stock I	500ml	200ml	100ml	50ml
Stock II	50ml	20ml	10ml	5ml
Stock III	50ml	20ml	10ml	5ml
Stock IV	50ml	20ml	10ml	5ml

Procedure for preparation of 1 liter tissue culture MS (1962) media :

Take 500ml double distill water (DDW) in a flask

↓

Added 50 ml of Stock-I

↓

Added 5ml of Stock-II

↓

Added 5ml of Stock-III

↓

Added 5ml of Stock-IV

↓

Added 30gm of Sucrose and dissolve it

↓

Added required amount of PGR

↓

Auxins was dissolved in alcohol and Cytokines were dissolved in NaOH

↓

Make up the volume up to 1000ml with DDW

↓

Maintained the pH of solution with 1N HCl and 1N NaOH, it was 5.7-5.8

↓

Added 8.0gm agar in the medium, melted it in microwave oven

↓

Poured the media into test tubes and bottles and were autoclaved for 30 minutes.

Vertical autoclave used for media preparation

Methods for fresh culturing and sub culturing:

All the inoculations were made aseptically under a laminar airflow hood. Before initiating the process of inoculation, laminar airflow hood was sterilized by switching on UV radiations for 30-40 minutes and the surface of the laminar air flow working table was cleaned by whipping it with 95% ethyl alcohol before initiating any operation under the hood. The ultra clean air blown through HEPA (high efficiency particulate air) filters,

being free from fungal and bacterial contaminants made the transfer of plant material into the culture vials very safe.

The instruments like foreceps, the scalpel blade and scissor etc. were flame sterilized before using them for the inoculation. The hands were cleaned by 90% ethyl alcohol, mouth and nose were covered with a mask so as to avoid the contamination of the cultures.

Method of Fresh Culturing (Open Lab):

Collection of explants from the field

⇓

Kept it in a bottle covered with muslin cloth

⇓

Keep it under running tap water to remove dirt and pathogens for 30minutes.-45 minutes.

⇓

Washed it 4-5 times with Double Distill Water (DDW)

⇓

Treated the explants with extran (2%) for 15 minutes to kill the bacteria and other pathogens

⇓

Washed it 5-6 times with DDW

⇓

Now treated the explants with (2%) Bevistin solution for 10-15 minutes

⇓

Washed it 4-5 times with DDW for the complete removal of Bevistin

⇓

Transferred the bottle to Laminar Air Flow chamber

Close Lab -

Wiped LAF and glass wares with alcohol

⇓

LAF cabinet and glass wares were sterilized under UV light for at least 45 minutes-1hour

⇓

Kept the washed explants and all needed apparatus like sterile DDW bottles, required media, forceps, and scalpel; under UV light in LAF for 40 minutes

⇓

Now transferred it in pre-sterilized bottle with the help of pre-sterilized forceps help of pre-sterilized forceps

⇓

Washed it with 4-5 times with sterilized DDW

⇓

Added 0.1% $HgCl_2$ solution and keep it for 2-3 minutes (time may vary with the explants)

⇓

Washed it 4-5 times with sterile DDW again

⇓

Explants were ready for inoculation

Inoculation Method -

Take the material out with the help of sterile forceps into pre-sterilized bottle with cap

⇓

Transfer it in a petriplate

⇓

Trim both the ends of the explants

⇓

Inoculate the explants into the culture media (tube) over the flame

⇓

Covered it with the cap or appropriate closure

⇓

Sealed it with tape

⇓

Labeled the culture vessels with the name of the explants and the date of inoculation

⇓

Material thus inoculated were kept in the culture room at 25+2°C

Inoculation method of sub culturing -

Take out the explants from test tube with the help of sterile forcep.

⇓

Transfer it in a petripate.

⇓

With the scalpel, trim the ends of explants.

⇓

Inoculated the explants into the bottles containing the culture media over the flame.

⇓

Covered the bottles with the caps.

⇓

Sealed it with tapes.

⇓

Labelled the culture vessel with the name and date of inoculation.

⇓

Materials thus inoculated were kept in a culture room at 25±2°C.

c. Maintenance of cultures in culture room

After inoculation, the cultures were transferred to the culture room which was initially sterilized by washing all of its walls and floor. The cleaning of culture room was done at regular intervals. Initially culture vials containing the plant material were incubated at 25±2°C temperature and 16 hours of photo period provided by PAR lamp and cool white florescent tube lights. Observations were made from 7th day onwards at weekly interval. The observation were recorded included different morphogenetic response in terms of formation and differentiation of organs, adventitious development of shoots and roots from the explants etc. After 5 weeks of inoculation sub-culturing was done at regular interval.

d. Hardening and acclimatization of plants:

After properly developed of plantlets, they were transferred to mist chamber for acclimatization and hardening. Rooted plants were removed from the medium, agar sticking to their roots washed with tap water, and they were transplanted into small size of polythene bags containing a suitable potting mixture (1:1:1) sand, soil and FYM. Plants were kept in high (90% or more) humidity and low light intensities. High humidity was maintained by covering the plants with plastic bags or plastic sheets. The humidity was gradually decreased to ambient level after about 7-15 days, and the light intensity was increased. The plants were then finally exposed to glass house conditions.

Following experimental designs were laid out for obtaining morphogenetic response in terms of shoot multiplication as well as induction of root from *in vitro* grown plants.

Experimental design for *in vitro* multiplication on MS culture media with BAP and IAA of designated species

PGRs	Treatments	PGR concentration (mg/lit.)
No PGRs	T0	No PGRs
MS+BAP	T1	0.1
	T2	0.2
	T3	0.3
	T4	0.4
	T5	0.5
	T6	1.0
	T7	2.0
	T8	3.0
	T9	4.0
	T10	5.0
MS+IAA+BAP	T11	0.1+1.0
	T12	0.1+2.0
	T13	0.1+3.0

Table cont...

	T14	0.1+4.0
	T15	0.1+5.0
	T16	0.5+1.0
	T17	0.5+2.0
	T18	0.5+3.0
	T19	0.5+4.0
	T20	0.5+5.0
	T21	1.0+2.0
	T22	1.0+3.0
	T23	1.0+4.0
	T24	1.0+5.0
	T25	2.0+2.0
	T26	2.0+3.0
	T27	2.0+4.0
	T29	2.0+5.0
	T30	3.0+3.0
	T31	3.0+4.0
	T32	3.0+5.0

Number of replications - 3 (R1, R2, R3)
Number of test tubes for each replications – 10

Experimental design for *in vitro* multiplication of designated species on MS Culture Media with PGR Auxin (IAA) and Cytokinin (Kn)

PGRs	Treatments	PGR concentration (mg/lit.)
No PGRs	**T0**	**No PGRs**
MS+Kn	T1	0.1
	T2	0.2
	T3	0.3
	T4	0.4
	T5	0.5
	T6	1.0
	T7	2.0
	T8	3.0
	T9	4.0
	T10	5.0
MS+IAA+Kn	T11	0.1+1.0
	T12	0.1+2.0
	T13	0.1+3.0
	T14	0.1+4.0

Table cont...

	T15	0.1+5.0
	T16	0.5+1.0
	T17	0.5+2.0
	T18	0.5+3.0
	T19	0.5+4.0
	T20	0.5+5.0
	T21	1.0+2.0
	T22	1.0+3.0
	T23	1.0+4.0
	T24	1.0+5.0
	T25	2.0+2.0
	T26	2.0+3.0
	T27	2.0+4.0
	T29	2.0+5.0
	T30	3.0+3.0
	T31	3.0+4.0
	T32	3.0+5.0

Number of replications - 3 (R1, R2, R3)

Number of test tubes for each replications - 10

Experimental design for *in vitro* multiplication of designated species on MS Culture Media with PGR Auxin (IBA) and Cytokinin (BAP)

PGRs	Treatments	PGR concentration (mg/lit.)
No PGRs	T0	No PGRs
MS+IBA	T1	0.1
	T2	0.2
	T3	0.3
	T4	0.4
	T5	0.5
	T6	1.0
	T7	2.0
	T8	3.0
	T9	4.0
	T10	5.0
MS+IBA+BAP	T11	0.1+1.0
	T12	0.1+2.0
	T13	0.1+3.0
	T14	0.1+4.0
	T15	0.1+5.0

Table cont...

	T16	0.5+1.0
	T17	0.5+2.0
	T18	0.5+3.0
	T19	0.5+4.0
	T20	0.5+5.0
	T21	1.0+2.0
	T22	1.0+3.0
	T23	1.0+4.0
	T24	1.0+5.0
	T25	2.0+2.0
	T26	2.0+3.0
	T27	2.0+4.0
	T29	2.0+5.0
	T30	3.0+3.0
	T31	3.0+4.0
	T32	3.0+5.0

Number of replications - 3 (R1, R2, R3)
Number of test tubes for each replications – 10

Experimental design for *in vitro* multiplication of designated species on MS Culture Media with PGR Auxin (NAA) and Cytokinin (BAP)

PGRs	Treatments	PGR concentration (mg/lit.)
No PGRs	**T0**	**No PGRs**
MS+NAA	T1	0.1
	T2	0.2
	T3	0.3
	T4	0.4
	T5	0.5
	T6	1.0
	T7	2.0
	T8	3.0
	T9	4.0
	T10	5.0
MS+NAA+BAP	T11	0.1+1.0
	T12	0.1+2.0
	T13	0.1+3.0
	T14	0.1+4.0
	T15	0.1+5.0
	T16	0.5+1.0

Table cont...

	T17	0.5+2.0
	T18	0.5+3.0
	T19	0.5+4.0
	T20	0.5+5.0
	T21	1.0+2.0
	T22	1.0+3.0
	T23	1.0+4.0
	T24	1.0+5.0
	T25	2.0+2.0
	T26	2.0+3.0
	T27	2.0+4.0
	T29	2.0+5.0
	T30	3.0+3.0
	T31	3.0+4.0
	T32	3.0+5.0

Number of replications - 3 (R1, R2, R3)
Number of test tubes for each replications – 10

Experimental design for *in vitro* rooting on MS Culture Media with PGR Auxin (IBA)

PGRs	Treatments	PGR concentration (mg/lit.)
No PGRs	**T0**	**No PGRs**
MS+IBA	T1	0.1
	T2	0.2
	T3	0.3
	T4	0.4
	T5	0.5
	T6	1.0
	T7	2.0
	T8	3.0
	T9	4.0
	T10	5.0

Number of replications - 3 (R1, R2, R3)
Number of test tubes for each replications – 10

e. Methods for clonal macropropagation of *Butea superba*

(i) Preparation of cuttings for macropropagation:

The stem branch cuttings of *Butea superba* were collected carefully from different forest areas of Madhya Pradesh. The cuttings were properly collected in gunny bags and the moisture was maintained around the cuttings. The cuttings were brought in the mist chamber for further propagation. Different length sizes of cuttings (10-15cm) with different thickness (2 to 3cm) were prepared alongwith at least one or two nodes for rooting of cuttings through macropropagation protocol.




Collection of cuttings from wild sources

(ii) Treatment of cuttings

Different ppm concentrations of root promoting hormones viz Naphthalene Acetic Acid (NAA) and Indole-3-Butyric Acid (IBA) were prepared for optimizing the maximum root induction from the cuttings as mentioned below table.

Hormonal concentrations:

Name of root promoting hormones	Ranges (ppm)	Company name	Time of treatment of cuttings
Indol-3 Butyric Actic -(IBA)	100 to 2000	Sigma	10 to 35 min
α-Naphthalene Acetic Acid (NAA)	100 to 2000	Sigma	10 to 35 min

(iii) Placement of cuttings under mist chamber and polypropagator –

The treated cuttings were placed horizontally and vertically in mist chamber and polypropagators on medium grade pure sand with following congenial physical conditions:

a. Temperature: 35°to 45°C.

b. Humidity: 80 to 90% with intermittent misting.

c. Spraying frequency 3 to 4 times in summer and 2 to 3 times in other seasons.

d. 10 cuttings in 3 replications (30 cuttings) of each sub treatment were tried for standardization of macropropagation protocol as mentioned below Table.

Experimental design for rooting of cuttings of Butea superba in 3 replication (R1, R2, R3) for each sub treatment:

Hormones	Hormonal concentration (ppm)	Hormonal Treatments	Sub-treatments for timing in minutes			
			10	20	30	40
Control	–	T0				
	100	T1	T1a	T1b	T1c	T1d
	200	T2	T2a	T2b	T2c	T2d
	500	T3	T3a	T3b	T3c	T3d
	1000	T4	T4a	T4b	T4c	T4d
	1500	T5	T5a	T5b	T5c	T5d
	2000	T6	T6a	T6b	T6c	T6d

(iv) Observations -

Observation were made for recording the root induction from the cuttings with following parameters.

i. No. of days for rhizogenesis and celogensis.

ii. Type of cuttings showed rooting response.

iii. Number of roots and its length.

iv. Appropriate hormonal concentration.

v. Appropriate time of treatment of cuttings.

Chapter-4

Result and Discussion

A. Macropropagation of *Alectra chitrakutensis*:

The species is host specific parasitic in nature and grows alongwith the roots of Nirgundi (*Vitex Negundo* L.) hence, its macropropagation through stem cuttings were very difficult. The stem cuttings was treated with different ppm concentrations of IBA and NAA (100-2000 ppm) for different time duration (10-30 minutes). It was noticed that the hormonal treatment of IBA and NAA have no significant effects for producing roots from stem cuttings. The stem cuttings were dried after 15-20 days. This may be the parasitic nature of plant with specific host *Vitex Negundo* Then the rooting were induced in the cuttings of *Vitex Negundo* and then the stem cuttings alongwith rhizomes of *Alectra chitrakutensis* were shifted with rooted cuttings of host plant. It was observed that the new plants of *Alectra chitrakutensis* were emerged during November to December and were full grown during March to April.

B. Macropropagation of *Butea superba*:

The result of the present study are given in below table which reveal that the Auxin, Indole-3-butyric acid (IBA) and Naphthaleneacetic acid (NAA) showed that the concentrations and time differed significantly from each other in respect of percentage of rooting of cuttings. The rooting initiation in the cutting were started within 20 to 25 days in the month of April to June. It was noticed that during Feb to April the rooting was initiated after 30 to 35 days. The rooting responses in (Horizontal and Vertical) cuttings are given in following tables respectively. It was observed that the horizontally placed cutting showed maximum rooting response than the vertically placed cutting under mist chamber and polypropagator. The maximum rooting response was observed as 26% in horizontally placed cuttings when they were treated with 100ppm concentration solution of NAA for 10 minutes in 30 to 35 days, while IBA showed poor rooting response (19%) with same concentration. The other concentration of NAA and IBA showed poor to moderate rooting response. A large number of workers reported that hardwood species are difficult to propagate by vegetative means because they are recalcitrant in nature (Anon,1988; Barnes and Busly 1987; Charturvedi, 1983 and Tiwari; *et.al.* 1998, Ram Prakash *et.al.* 2014). So, this macropropagation protocol can helps to multiply and produce true to type clones of this species.

Effect of different ppm concentration of NAA and time on the rooting percentage in horizontally placed cuttings for 30 to 35 days.

Treatments NAA	Time of treatment (Minutes)	No. of root/ cutting	Root length (in cm)	% of rooting
T1 (100ppm)	**10**	**2**	**20-25**	**26**
	20	2	10-15	18
	30	Nil	Nil	Nil
	40	Nil	Nil	Nil
T2 (200ppm)	10	Nil	5-10	5
	20	Nil	Nil	Nil
	30	Nil	Nil	Nil
	40	Nil	Nil	Nil
T3 (500ppm)	10	Nil	3-6	2
	20	Nil	Nil	Nil
	30	Nil	Nil	Nil
	40	Nil	Nil	Nil
T4 (1000ppm)	10	Nil	Nil	Nil
	20	Nil	Nil	Nil
	30	Nil	Nil	Nil
	40	Nil	Nil	Nil
T5 (1500ppm)	10	Nil	Nil	Nil
	20	Nil	Nil	Nil
	30	Nil	Nil	Nil
	40	Nil	Nil	Nil
T6 (2000ppm)	10	Nil	Nil	Nil
	20	Nil	Nil	Nil
	30	Nil	Nil	Nil
	40	Nil	Nil	Nil

Effect of different ppm concentration of NAA and time on the rooting percentage in vertically placed cuttings for 30 to 35 days.

Treatments IBA	Time of treatment (Minutes)	No. of root/ cutting	Root length (in cm)	% of rooting
T1 (100ppm)	**10**	**1**	**10-15**	**19**
	20	1	8-10	13
	30	Nil	Nil	Nil
	40	Nil	Nil	Nil
T2 (200ppm)	10	Nil	Nil	Nil
	20	1	5-6	6
	30	Nil	Nil	Nil
	40	Nil	Nil	Nil
T3 (500ppm)	10	Nil	Nil	Nil
	20	Nil	Nil	Nil
	30	Nil	Nil	Nil
	40	Nil	Nil	Nil
T4 (1000ppm)	10	Nil	Nil	Nil
	20	1	3-5	3
	30	Nil	Nil	Nil
	40	Nil	Nil	Nil
T5 (1500ppm)	10	Nil	Nil	Nil
	20	Nil	Nil	Nil
	30	Nil	Nil	Nil
	40	Nil	Nil	Nil
T6 (2000ppm)	10	Nil	Nil	Nil
	20	Nil	Nil	Nil
	30	Nil	Nil	Nil
	40	Nil	Nil	Nil

Effect of different ppm concentration of IBA and time of rooting % in horizontally placed cuttings for 30 to 35 days.

Hormonal Treatments IBA	Time of Treatment (Minutes)	No.of root/ cutting	Root length (in cm)	% of rooting
T1 (100ppm)	**10**	**1**	**10-15**	**19**
	20	1	10-12	13
	30	Nil	Nil	Nil
	40	Nil	Nil	Nil
T2 (200ppm)	10	Nil	Nil	Nil
	20	Nil	Nil	Nil
	30	Nil	Nil	Nil
	40	Nil	Nil	Nil
T3 (500ppm)	10	Nil	Nil	Nil
	20	1	5-6	5
	30	Nil	Nil	Nil
	40	Nil	Nil	Nil
T4 (1000ppm)	10	Nil	Nil	Nil
	20	Nil	Nil	Nil
	30	Nil	Nil	Nil
	40	Nil	Nil	Nil
T5 (1500ppm)	10	Nil	Nil	Nil
	20	Nil	Nil	Nil
	30	Nil	Nil	Nil
	40	Nil	Nil	Nil
T6 (2000ppm)	10	1	3-5	3
	20	Nil	Nil	Nil
	30	Nil	Nil	Nil
	40	Nil	Nil	Nil

Effect of different ppm concentration of IBA and time on the rooting percentage in vertically placed cuttings for 30 to 35 days.

Hormonal Treatments IBA	Time of treatment (Minutes)	No. of root/ cutting	Root length (in cm)	% of rooting
T1 (100ppm)	**10**	**1**	**10-15**	**17**
	20	1	8-10	10
	30	Nil	Nil	Nil
	40	Nil	Nil	Nil
T2 (200ppm)	10	Nil	Nil	Nil
	20	Nil	Nil	Nil
	30	Nil	Nil	Nil
	40	Nil	Nil	Nil
T3 (500ppm)	10	Nil	Nil	Nil
	20	1	3-4	4
	30	Nil	Nil	Nil
	40	Nil	Nil	Nil
T4 (1000ppm)	10	Nil	Nil	Nil
	20	Nil	Nil	Nil
	30	Nil	Nil	Nil
	40	Nil	Nil	Nil
T5 (1500ppm)	10	Nil	Nil	Nil
	20	Nil	Nil	Nil
	30	Nil	Nil	Nil
	40	Nil	Nil	Nil
T6 (2000ppm)	10	Nil	Nil	Nil
	20	Nil	Nil	Nil
	30	Nil	Nil	Nil
	40	Nil	Nil	Nil

C. Micropropagation of *Alectra chitrakutensis*:

Alectra chitrakutensis is critically endangered parasitic plant grows alongwith the roots of Nirgundi known as *Vitex Negundo* L.. *Alectra chitrakutensis* is a host specific parasite. Its macropropagation through stem rhizomes has already been described above. Attempts have been made for standardization its micropropagation protocol. The stem cuttings alongwith small rhizomes were inoculated as a source of explants on MS culture media without host plants. The results which are obtained are described in the following table.

Morphogenetic response of BAP and Kn on stem with small rhizome explants of *Alectra chitrakutensis* on MS culture media (Culture period 40 to 45 days)

Cytokinins (mg/lit.)	No. of shoots/ explants	Shoot length (cm)	% Response
Control	0.50±0.32	0.92±0.21	30.33
BAP (0.5)	1.20±0.33	1.94±0.29	53.33
BAP (1.0)	1.73±0.60	2.48±0.44	60.00
BAP (2.0)	2.26±0.48	2.86±0.59	63.33
BAP (3.0)	**3.06**±0.83	**4.30**±0.70	**76.66**
BAP (4.0)	2.46±0.48	3.79±0.65	73.33
BAP (5.0)	2.20±0.73	2.66±0.58	73.33
Kn (0.5)	0.60±0.30	0.42±0.21	25.00
Kn (1.0)	1.10±0.32	1.34±0.23	40.00
Kn (2.0)	2.33±0.62	2.38±0.42	60.00
Kn (3.0)	**3.00**±0.81	**3.30**±0.71	**73.33**
Kn (4.0)	2.06±0.83	3.21±0.20	72.00
Kn (5.0)	2.46±0.48	3.79±0.65	72.00
Mean ± SD in each column fallowed by same letters at superscripts are not significantly different P < 0.05. (5 explants per treatment with 3 replication)			

BAP = 6-Benzylaminopurine, Kn = Kinetin

Alectra chitrakutensis showed excellent morphogenetic in terms of number of shoot/explants, shoot length and % response when the culture medium was supplemented 3.0mg/lit. BAP within 40 to 45 days of culture period with 76.66% response. The new shoots were emerges from the base of the adjoining areas of shoot and rhizome they were green and healthy. Like wise another cytokine Kn was also tested for morphogenetic response in this species it was observed that 3.0mg/lit. Kn

showed 3 shoots/explants with 3.30cm shoot length with 73.33% response. Comparatively BAP was found to be best Cytokinin as compare to Kn.

Effect of Auxine and Cytokinin on stem with small rhizome explants of *Alectra chitrakutensis* on MS culture media (Culture period 35 to 40 days)

Cytokinins (mg/lit.)	No. of shoots/ explants	Shoot length (cm)	% Response
IAA+ BAP (0.5+1.0)	1.13±0.29	1.88±0.24	53.33
IAA+ BAP (0.5+2.0)	1.66±0.51	2.56±0.37	66.66
IAA+ BAP (0.5+3.0)	**3.00** ±0.87	**4.30**±0.70	**76.66**
IAA+ BAP (0.5+4.0)	2.66±0.76	3.91±0.77	73.33
IAA+ BAP (0.5+5.0)	2.33±0.48	3.53±0.77	73.33
IAA+ Kn (0.5+1.0)	1.0±0.21	1.34±0.24	40.00
IAA+ Kn (0.5+2.0)	1.60±0.21	2.16±0.31	60.66
IAA+ Kn (0.5+3.0)	**2.66** ±0.82	**3.30**±0.77	**73.33**
IAA+ Kn (0.5+4.0)	2.33±0.76	3.01±0.37	66.66
IAA+ Kn (0.5+5.0)	2.30±0.42	2.53±0.24	66.66

Mean ± SD in each column fallowed by same letters at superscripts are not significantly different P < 0.05. (5 explants per treatment with 3 replication)

BAP = 6-Benzylaminopurine, Kn = Kinetin

When the culture medium supplemented with a combination of Auxin and Cytokinin it was observed there were no signification observation recorded in terms of number of shoots per explants, shoot length and % response when the media was supplemented with IAA 0.5+ BAP 3.0 mg/lit. A combination of IAA and Kn showed poor morphogenetic response in terms of number of shoot/explants, shoot length and % response. The similar work has been reported by Ahuja *et.al.* 1988, Mathur *et.al.* 1989 and Tiwari *et.al.* 1998 on other species.

Effect of Auxine and Cytokinin on stem with small rhizome explants of *Alectra chitrakutensis* on MS culture media (Culture period 40 to 45 days)

Cytokinins (mg/lit.)	No. of shoots/ explants	Shoot length (cm)	% Response
IAA+ BAP (1.0+1.0)	1.03±57	0.57±0.76	66.66
IAA+ BAP (1.0+2.0)	2.06±0.66	2.50±0.53	73.33
IAA+ BAP (1.0+3.0)	**3.33**±0.64	**4.70**±0.36	**83.33**
IAA+ BAP (1.0+4.0)	2.40±0.51	3.89±0.48	73.33
IAA+ BAP (1.0+5.0)	2.33±0.69	2.86±0.34	73.33
IAA+ Kn (1.0+1.0)	1.00±52	0.50±0.76	66.66
IAA+ Kn (1.0+2.0)	2.00±0.61	2.20±0.53	73.33
IAA+ Kn (1.0+3.0)	**3.30**±0.61	**3.90**±0.36	**76.66**
IAA+ Kn (1.0+4.0)	2.20±0.50	3.39±0.42	73.33
IAA+ Kn (1.0+5.0)	2.13±0.61	2.46±0.31	66.66

Mean ± SD in each column fallowed by same letters at superscripts are not significantly different P < 0.05. (5 explants per treatment with 3 replication)

Effect of Auxine and Cytokinin on stem with small rhizome explants of *Alectra chitrakutensis* on MS culture media (Culture period 40 to 45 days)

Cytokinins (mg/lit.)	No. of shoots/ explants	Shoot length (cm)	% Response
IAA+ BAP (2.0+1.0)	0.53±51	0.50±0.72	55.33
IAA+ BAP (2.0+2.0)	1.06±0.33	1.50±0.53	66.66
IAA+ BAP (2.0+3.0)	**2.33**±0.63	**2.54**±0.33	**73.33**
IAA+ BAP (2.0+4.0)	2.30±0.50	2.19±0.42	73.33
IAA+ BAP (2.0+5.0)	1.33±0.62	1.82±0.32	55.33
IAA+ Kn (2.0+1.0)	0.40±52	0.50±0.72	66.66
IAA+ Kn (2.0+2.0)	1.00±0.62	1.20±0.52	73.33
IAA+ Kn (2.0+3.0)	**2.10**±0.61	**2.30**±0.33	**73.33**
IAA+ Kn (2.0+4.0)	1.80±0.50	2.32±0.40	73.33
IAA+ Kn (2.0+5.0)	1.13±0.33	1.46±0.33	55.33

Mean ± SD in each column fallowed by same letters at superscripts are not significantly different P < 0.05. (5 explants per treatment with 3 replication)

From above tables it was observe that by increasing the concentration of IAA 1.0 mg/lit. and 2.0 mg/lit. alongwith BAP and Kn separately showed no significant variation in terms of number of shoots/explants, shoot length and % response. Therefore, BAP at 3.0 mg/lit. showed excellent morphogenetic response in terms of number of shoot/ explants, shoot length and % response. From the above findings It is noticed that this species is a host specific in nature and without host it could not grow in nature but under micro-propagation study it grows without host under *in vitro* condition. The micropropagated shoots were harvested and shifted with host plants under field condition and it was observed that within 15 days interval the new plants were emerges with host. So this *in vitro* studies is very important for conservation and multiplication of this critically endangered species.

D. Micropropagation of *Butea superba*:

Butea superba is rare medicinal plant. Its cloning through macropropagation has already been described above with 26% success rate through stem branch cuttings. An attempt have been made for its large scale multiplication through tissue culture technique. The new sprouts of nodal segments from stem branch cuttings were obtained from mist chamber during February and March. The nodal segments were inoculated on MS (1962) culture medium with various combination and concentration of auxins and cytokinins. The results which were obtained are described in below tables –

Effect of only BAP and Kn on morphogenetic response of shoots from nodal explants of *B.superba* (period 35 to 40 days) -

MS + Cytokinin (mg/lit.)	No. of shoots /explants	Shoot length (cm)	% Response
Control	Nil	Nil	00
BAP (4.0)	2.23±67	0.57±0.66	20
BAP (5.0)	4.66±0.80	1.16±0.18	40
BAP (6.0)	**5.6±0.83**	**3.42±0.36**	60
BAP (7.0)	3.73±0.45	2.16±0.23	49
BAP (8.0)	2.33±0.65	1.00±0.63	40
Kn (4.0)	1.03±57	0.51±56	18
Kn (5.0)	4.06±0.70	1.06±0.28	40
Kn (6.0)	**5.0±0.83**	**3.22±0.56**	56
Kn (7.0)	3.13±0.55	2.06±0.63	49
Kn (8.0)	2.43±0.58	1.06±0.83	41
Mean ± SD in each column fallowed by same letters at superscripts are not significantly different P < 0.05 . (5 explants per treatment with 3 replication)			

BAP = 6-Benzylaminopurine, Kn = Kinetin

From the above study, it was observed that when MS culture media was supplemented alone with 6.0mg/lit. of BAP showed maximum shoot morphogenetic response with 60% success within 35 to 40 days of culture period. On the other hand an another group of cytokinin Kn at 6.0mt/lit. showed 56% morphogenetic response from nodal explants in terms of shoot of growth. There are several literature and reviews are available that Cytokinin BAP is more effective Cytokinin as compare to Kn. The other concentration of both the Cytokinin, showed poor to moderated morphogenetic response in terms of shoot growth. In this observation an interesting phenomena was also observed that most of the plant species showed excellent morphogenetic response in low concentration of plant growth regulators (PGR 0.5 to 5.0 mg/lit.). While in case of *Butea superba* showed excellent morphogenetic response in terms of shoot multiplication when the cytokinin concentration kept higher range (6.0 to 10.0 mg/lit.). This may be due to the recalcitrant nature of propagation of this species through micropropagation.

Effect of a combination of Auxins and Cytokinin (IAA+ BAP and IAA+ Kn) on morphogenetic response of shoots from nodal explants of *B.superba* (period 35 to 40 days) –

MS + Cytokinin (mg/lit.)	No. of shoots /explants	Shoot length (cm)	% Response
Control	Nil	Nil	00
IAA+ BAP (0.5+4.0)	0.53±57	0.51±0.71	13
IAA+ BAP (0.5+5.0)	4.06±0.50	1.21±0.51	35
IAA+ BAP (0.5+6.0)	**4.6±0.51**	**3.56±0.52**	**55**
IAA+ BAP (0.5+7.0)	3.03±0.45	2.34±0.60	43
IAA+ BAP (0.5+8.0)	1.03±0.61	1.20±0.30	34
IAA+ Kn (0.5+4.0)	1.00±51	0.50±50	11
IAA+ Kn (0.5+5.0)	3.06±0.50	1.00±0.40	33
IAA+ Kn (0.5+6.0)	**3.00±0.41**	**2.62±0.51**	**51**
IAA+ Kn (0.5+7.0)	3.00±0.50	2.54±0.50	42
IAA+ Kn (0.5+8.0)	1.03±0.50	1.00±0.40	26

Mean ± SD in each column fallowed by same letters at superscripts are not significantly different P < 0.05. (5 explants per treatment with 3 replication)

BAP = 6-Benzylaminopurine, Kn = Kinetin

From the above table a combination of Auxin and Cytokinin showed that the number of shoots per explants were reduced (4.6) while the shoot length were increased (3.56 cm) when the culture media was supplemented with IAA+BAP (0.5+6.0 mg/lit.). As per the available literature IAA is a Auxine which help to increase cell length. Here in this table from the observation it is clear that IAA plays a vital role to increase the shoot length. On the other hand a combination of IAA and Kn (0.5+6.0) lowering the number of shoot as well as IAA has also not found effective to increase cell length in terms of shoot length.

Effect of Auxins and Cytokinin (IAA+ BAP and IAA+ Kn) on morphogenetic responseof shoots from nodal explants of *B.superba* (period 35 to 40 days)

MS + Cytokinin (mg/lit.)	No. of shoots /explants	Shoot length (cm)	% Response
Control	Nil	Nil	00
IAA+ BAP (1.0+4.0)	1.03±57	0.57±0.76	20
IAA+ BAP (1.0 +5.0)	4.06±0.50	1.06±0.58	38
IAA+ BAP (1.0+6.0)	**4.6±0.53**	**3.02±0.56**	**58**
IAA+ BAP (1.0+7.0)	3.23±0.45	2.10±0.63	47
IAA+ BAP (1.0+8.0)	2.03±0.65	1.00±0.33	38
IAA+ Kn (1.0+4.0)	1.00±57	0.50±51	15
IAA+ Kn (1.0+5.0)	4.06±0.50	1.00±0.20	36
IAA+ Kn (1.0+6.0)	**4.40±0.43**	**3.02±0.51**	**53**
IAA+ Kn (1.0+7.0)	3.10±0.50	2.00±0.60	44
IAA+ Kn (1.0+8.0)	2.03±0.50	1.00±0.43	31
Mean ± SD in each column fallowed by same letters at superscripts are not significantly different P < 0.05. (5 explants per treatment with 3 replication)			

BAP = 6-Benzylaminopurine, Kn = Kinetin

It was observed from above table that when the IAA (1.0mg/lit.) concentration was increased with BAP (1.0+6.0 mg/lit.) the number of shoots per explants were reduced as well as shoot length was also reduced with 58% response the similar phenomenon was observed with a combination IAA + Kn (1.0+6.0 mt/lit.) with 53% response.

Effect of Auxins and Cytokinin (IAA+ BAP and IAA+ Kn) on morphogenetic response of shoots from nodal explants of *B.superba* (period 35 to 40 days)

MS + Cytokinin (mg/lit.)	No. of shoots /explants	Shoot length (cm)	% Response
Control	Nil	Nil	00
IAA+ BAP (2.0+4.0)	0.43±51	0.50±0.70	10
IAA+ BAP (2.0+5.0)	2.16±0.52	1.16±0.52	30
IAA+ BAP (2.0+6.0)	**2.6±0.51**	**2.02±0.50**	**50**
IAA+ BAP (2.0+7.0)	2.21±0.41	1.60±0.60	41
IAA+ BAP (2.0+8.0)	2.00±0.61	1.00±0.30	33
IAA+ Kn (2.0+4.0)	0.40±52	0.40±51	10
IAA+ Kn (2.0+5.0)	2.06±0.51	1.00±0.50	32
IAA+ Kn (2.0+6.0)	**2.34±0.45**	**2.12±0.53**	**46**
IAA+ Kn (2.0+7.0)	2.11±0.50	1.60±0.60	40
IAA+ Kn (2.0+8.0)	1.03±0.53	0.60±0.43	25

Mean ± SD in each column fallowed by same letters at superscripts are not significantly different P < 0.05. (5 explants per treatment with 3 replication)

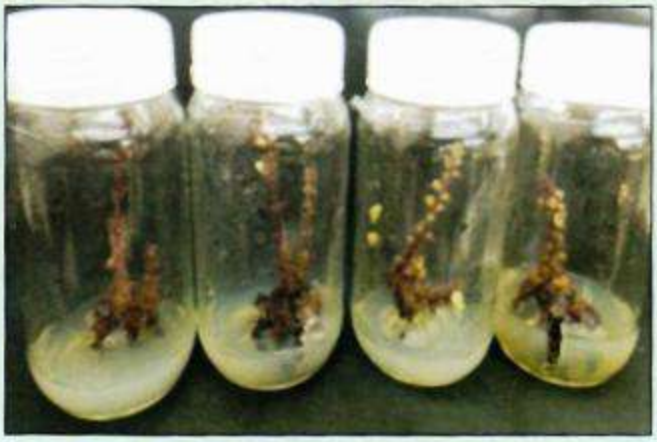
BAP = 6-Benzylaminopurine, Kn = Kinetin

As the concentration of IAA (2.0mg/lit.) increased with both BAP and Kn showed poor morphogenetic response in terms of number of shoots per explants, shoot length and % response. So, from the above tables it is observed that BAP alone (6.0 mg/lit.) or combination with IAA (0.5+6.0 mg/lit.) showed excellent morphogenetic response in terms of number of shoot per explants, shoot length and % response.

E. In vitro rooting of *Butea superba* :

An attempt have been made for the induction of *in vitro* rooting from the *in vitro* grown shoots of this species. When the MS Culture medium was supplemented with IBA and NAA (0.5mg/lit. to 5.0mg/lit.) showed no rooting response from *in vitro* grown shoot. Higher the concentration of PGR showed yellowing of shoots. The *in vitro* shoot were harvested and shifted for rooting in mist chamber on medium grade sand. Very poor rooting response were observed from *in vitro* regenerated shoots. The hardened plants were showed poor survival rate.

F. Photo Plates



Micropropagation of *Alectra chitrakutensis*

In vitro grown plants of *Alectra chitrakutensis* with host plants

Horizontal cuttings of *Butea superba* in mist chamber

Induction of root of horizontal cuttings in *Butea superba*

In vitro regeneration of *Butea superba*

G. Discussion

Conservation and propagation of plant species is one of the thrust area of research particularly for rare, endangered and threaten medicinal plant. Several biotechnological interventions are now available for conservation and multiplication of such taxa. In this bulletin two propagation technology have been standardized so far for multiplication purpose. After evaluation of quality planting material have been selected for multiplication through macro and micro propagation technique. In case of *Butea superba* the rooting response were observed both from horizontally and vertically placed cuttings under mist chamber. It is concluded that the horizontally placed cuttings showed maximum 26% rooting response when they were treated with NAA (100ppm) for 10 minutes under mist chamber/poly propagators from April to June. On the other

hand 19% rooting response were observed when the cuttings were treated with IBA at 100ppm. So, from the above finding it is concluded that NAA at 100ppm for 10 minute treatment during April to June is a most appropriate root promoting hormone for this species. Similarly, the macropropagation of *Alectra chitrakutensis* have been also tried but it was very difficult to propagate it through stem cuttings while IBA and NAA root promoting hormone were tried from 100ppm to 2000ppm. This may be due to the parasitic nature of this species. Then it was concluded that the rooted cuttings of *Vitex Negundo* L. supports the growth of stem cuttings of this species under field condition. So, macropropagation of this species through stem branch cuttings was not found successfully. Another micropropagation method of propagation of designated species were also tried for their multiplication. It is concluded that when the MS Culture medium was supplemented with BAP 6.0mg/lit the nodal explants of *Butea superba* showed excellent morphogenetic response in terms of shoot multiplication and shoot length. However, the *in vitro* regenerated shoot were failed to produce *in vitro* rooting. The *in vitro* regenerated shoots were showed poor rooting response under *ex vitro* condition. Similarly, the stem rhizome of *Alectra chitrakutensis* showed excellent morphogenetic response in terms of shoot multiplication when the MS/Culture medium supplemented with BAP 3.0mg/lit. The *in vitro* regenerated shoots were successfully hardened alongwith the roots of host plant under field condition. So, by this way critically endangered species can easily be multiplied and conserved.

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