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PROPAGATION OF MEDICINAL PLANTS, BAMBOO AND RATTAN BY TISSUE CULTURE

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ABBREVIATIONS

BAP		**Benzyl amino purine**
CH	–	**Casein hydro1ysate**
2,4-D	–	**2,4-Dichlorophenoxyacetic acid**
Kin	–	**Kinetin**
IBA	–	**Indole butyric acid**
mg/l	–	**milligrams per Liter**
NAA	–	**Naphthalene acetic acid**
v/v	–	**volume /volume**
w/v	–	**weight/volume**

ABSTRACT

Tissue culture of seiected species of medicinal plants, bamboos and rattans were carried out to develop methods for micrpropagation. In two bamboo species viz. *Bambusa bambos* and *Dendrocalamus hamiltonii,* successful micropropagation was carried out using seedlings. Multiplication of shoots and rooting was obtained on a cytokinin containing medium. Shoot multiplication was higher in liquid medium than on solid medium. *In vitro* flowering was also observed in shoot cultures of *B. bambos.* Nodal exalants taken from mature culms of *B. bambos, B. bambos var. gigantea, B. vulgaris* and *Thyrsostachys oliverii* formed multiple shoots on cytakinin containing media but further maintenance of shoot cultures or rooting of the shoots was not obtained.

Micropropagation was carried out in three species of rattans **viz.** -*CaIamus thwaitesi I, C. pseudatenuis* and *C. hookerianus.* from embryos isolated from seeds. Shaot multiolication was obtained on a solid medium containing cytokinins. Optimal multiplication was obtained on **a** medium supplemented with 3 mg/l of Kin and 1 mg/l of BAP. Multiplication was also oossible in a liauid medium without shaking. Rooting was induced by transfer of shoots to a medium containing 1 mg,/l IBA for 48 hrs followed by a hormone free medium. Plantlets transferred to a soil-vermiculite (2:1) mixture and hardened in a mist propagation chamber for 8 weeks, survived and continued to grow. Plantlets with 3-6 shoots could be produced by rooting? entire clusters of multiple shoots in *C. thwaitessi.* Somatic embryos and piantlets were also induced in cultures of *C. hookerianus.*

Micropropagation of a medicinal plant – *Kaempferia galanga* was done using rhizome buds. Shoot multiplication and rooting was obtained on media containing 0.5-5 **mg/l** of. BAP or Kin. More than 80 % of the plantlets transferred to soil survived without any hardeninq. Plantlets were grown in the field with control plants produced from rhizomes. After one year of growth, micropropagated plants produced rhizomes that were thinner and more branched than the controls, but the total rhizome weight per plant were simi1ar.

Micropropagation of *Malaxis rheedii* – a medicinal orchid was attempted from oseudobulbils. Prolific multiple shoot formation was obtained from axillary buds after 3-4 subcultures on cytok inin containing medium. Rooting was observed on a charcoal containinq medium after a brief auxin treatment, but plantlets did not survive. Micropropagation of a medicinal tree – *Wrightia tinctoria* was attempted from seedlings and leaf parts. Callus formation was induced on a wide range of auxin containing media but regeneration of plantlets was not obtained on any of the media tested.

1. INTRODUCTION

The use of tissue culture techniques for clonal propagation otherwise known as micropropagation has now been extended to a wide range of plants of economic importance. This includes trees, shrubs and herbaceous species. *In vitro* techniques have advantaqes like high multiplication rates, potential for production of disease free plants, plant production independent of season or time of the year and uniform plant quality. Micropropagation has now become the method of choice for propaqation of several important crops.

The plant groups selected for micropropagation in this study viz. bamboos, rattans and medicinal plants are of great relevance to the economy of Kerala and the well being of the people especially in the rural areas. A11 the three groups of plants have been affected by rapid loss of natural forests in recent decades in the State. Large scale planting of these plant5 will thus be necessitated if continued availability is to be ensured. However problem5 in mass propagation of these plants create hurdles in their cultivation on a big scale. An attempt to develop micropropagation methods for some important plant species belonging to three groups has been made in this study.

1.1. Bamboos

Bamboos are among the most important produce of the forests in Asia and the Pacific. The use of bamboos has traditionally been multifarious and it is the mainstay of several rural cottage industries. In more recent times bamboos have been used in huge quantities for paper-pulp manufacture. About 130 species of bamboos occur in India (Sharma, 1987). India has large reserves of bamboo and ranks second in the world in bamboo production (Pathak, 1989). *Bambusa* and *Dendrocalamus* are the most widely occurring genera, distributed throughout the peninsula to the Himalayan foothills. The important species of bamboos in Kerala are *Bambusa bambos* and ***Ochlandra*** spp.(reed bamboos). Several other species have potential for cultivation in the State.

Propagation of bamboos is carried nut either by seed or by use of vegetative propagation methods involving offsets, suckers or rhizomes (Mascarenhas and Muralidharan, 1993). While seeds are produced in huge amounts when certain species flower gregariously once in their long flowering cycles, these seeds lose their viability rapidly with the result that after a few years availability of seedling becomes difficult. In bamboos the solution has been to use a variety of vegetative propagation methods (Anonymous, 1990; Mascarenhas and Muralidharan, 1993). However, there are certain drawbacks in these methods that limit their use in mass propagation. Culm cuttinqs and rhizomes involve propagules that are bulky and difficult to transport. Until the formation of a rhizome takes place in 2-3 years, survival of the plant is poor unless care is taken. Multiplication rates are also limited by the number of cuttinqs that can be taken from the

The use of *invitro* techniques for propagation of bamboo has been reported in a large number of specie5 (Table 1.1). Most of the reports are those in which seeds or juvenile (seedling) parts have been used as the starting material. Induction of multiple shoots from nodes taken from seedlings followed by rooting of shoots is the most commonly used method in most of the reports.

Plantlet production through somatic embryogenesis has also been very successful in several species of bamboo. In *B. bambos* (Mehta *et al.*, *1982), D. strictus* (Rao *et* al., 1985) and *Sinocalamus latiflora* (Yeh and Chanq, 1987), somatic embryos were induced in callus obtained from seeds. Yeh and Chanq (1986, a, b.) also obtained somatic embryogenesis in callus derived from younq inflorescences of ***B.oldhamii*** and *B. beecheyana* respectively.

The induction of flowering in tissue cultures of bamboo derived from seedlings was reported in 3 species. Nadgauda *et al. (1990)* reported the phenomenon in *B. bambos, D. strictus* and *D. brandisii. In vitro* flowering in somatic embryos of *B. bambos* was also reported by Rao et *al.* (1988).

Micropropagation of mature bamboo using nodal explants has also been reported (Nadgir *et al.*, 1984). Bud break and multiple shoot formation from nodes of mature culms of *D. strictus* and *B. vulgaris* and low frequency of root formation was obtained.

In this project, two species viz. *B. bambos* and *D. hamiltonii* have been selected for micropropagation using seed and seedling parts. For experiments with explants collected from mature culms three species viz. *B. bambos var. gigantea, B. vulgaris* and *Thyrsostachys oliverii* were selected. Seed formation in the first two species ha5 never been observed and in the third flowering and seeding has not yet been obtained in Kerala.

Table 1.1 Previous work in tissue culture of Bamboos

Species	Explant	Results	References
B. bambos	Embryo	Callus, Som. embr.	Mehta et *al.,* 1982
	Nodes	Mult. Shoots	Nadgir *et al.,* 1984
B. beecheyana var. *beecheyana*	Infl.	Som. embr., Pl.	Yeh and Chang, 1986,b.
B.oldhamii	Shoot-tip	Callus, shoots	Huang, 1988
	Young Infl.	Som. embr., P1.	Yeh and Chang,l986,a.
B. vulgaris	Mature culm nodes	Mult. Shoots	Nadgir *et* a1.1984
D. hamiltonii	Seeds	Mult. Shoots, Pl.	Chambers *et al.*, 1991
D.strictus	Embryo	Callus, Som. embr. Pl.,	Rao et *al.,* 1985;
		Som. Emhr. Artificial seeds	Mukunthakumar & Mathur, 1992
	Seedling nodes	Mult. Shoots, PI.	Nadgir *et al.,1984*
	Mature culm nodes	Mult. Shoots, roots	Nadgir *et al.*, 1484
Phyl1ostachys viridis	Leaf	Callus, Som. embr. P1.	Hasan and Deberg, 1987
Sinocalamus latiflora	Embryo	Callus, Som. embr., P1.	Yeh and Chang, 1987

Embryo: Zygotic embryo; Infl.: Inflorescence; Som. embr.: Somatic embryo; Mult. Shoots: Multiple shoots; P1. : Plantlets;

1.2. Rattans

Rattans are a group of spiny, climbing or trailing palms of the tropical forests especially of South and South East Asia. Most rattans are monopodial ie. stems do not branch and there is a solitary growing point at the shoot tip. Many species however form clusters from the base of the stem after a few years of growth. The long jointed stems are used for furniture making and wickerwork and provides livelihood for millions of tribal and rural people in various parts of the world. India has about 60 species of rattans under 5 genera distributed mostly in the Western Ghats, the North Eastern states and the Andaman and Nicobar Islands (Renuka, 1995). Indiscriminate collection of raw canes from the forests; in the past has seriously depleted the rattan resources in the Western Ghats and the situation in other areas is also fast approaching this state. Plantations of rattan need to be established to counter this and ensure sustainable production in the future. KFRI has undertaken research in different aspects of rattan including propagation and cultivation methods.

Natural propagation of rattan is through seeds. Viability of rattan seeds is however short and germination is poor in soil. Removal of the fruit wall and pulp and sowing in moist saw dust enhances the germination rates. Vegetative propagation of rattan species is possible by removal of underground suckers but by this method only a limited number of plants can be produced. The potential for rapid large scale propagation of rattans by tissue culture has been discussed by Muralidharan (1992).

Table 1.2 summarizes the tissue culture research on rattans. Micropropagation of native rattan species have been carried out successfully in Malaysia and the Philippines. In seedling cultures of *C. manillensis,* multiplication of shoots and plantlet formation has been obtained by Patena et *al.* (1984). Yusoff (1989) obtained plantlets through callus and multiple shoots derived from embryos of C. *manan* and transferred them to soil. Aziah *et al.* (1985) obtained plantlets of *Calamus manan* from callus obtained from cultured embryos. Use of mycorrhizal inoculants to improve the survival and establishment of tissue cultured rattan plantlets has been obtained by Maziah (1991). Padmanaban and Ilangovan (1989) have grown whole plants through culture of zygotic embryos of *C.rotang.*

For the present study three rattan species of the Western Ghats in Kerala viz. *Calamus thwaitesii, C. pseudotenuis* and *C. hookerianus* have been selected. Tissue culture was attempted *by* usinq explants taken from immature or mature seeds and one year old seedlings.

Table 1.2 Previous work on tissue culture of rattan species

Species	Explant	Results	References
Calamus gamblei	Embryos	Multiple shoots	Padmanaban and Ilangovan (1994)
C. manillensis	Embryos	Plantlets	Patena et al. (1984)
C. manan	Embryo	Callus, Plantlets	Aziah et al. (1985) Gunawan and Yani (1986) Yusoff (1989)
	Seedling	Multiple shouts	Yusoff (1989)
C. rotang	Embryo	Germination, Plantlets	Padmanaban and Ilangovan

1.3. Medicinal Plants:

Medicinal plants have traditionally been collected from the wild. In recent times the increasing demand and indiscriminate collection has threatened many of these species. To ensure sustained production of these plants extensive cultivation is necessary. Difficulty in the propagation by seed or low multiplication rates in vegetatively propagated species is a hurdle for large scale cultivation. Tissue culture of several medicinal plants have been taken up to solve this problem.

The selection of medicinal plants for this study was based on their importance in Ayurvedic system of medicine. The plants were vegetatively propagated conventionally and multiplication rates were considered to be insufficient to meet the requirement for planting material for cultivation to be under taken on a Large scale.

Rhizomes of *Kaempferia galanga* (Family Zingeheraceae; Vernacular name: *Kacholam*) are highly aromatic and are used in around 80 different Ayurvedic formulations and as home remedies for stomach ailments. *K. galanga* became a highly remunerative crop in Kerala in recent years. Since the propagule (rhizome) is the commercial product, a demand for sufficient seed material exists. *Malaxis rheedii* (Family: Orchidaceae; Sanskrit: *Jeevakam)* is a terrestrial orchid that is becoming rare in the wild habitat, The fleshy psudobulbils of the plant forms one of the *Ashtavarga* - a group of eight plants used in Ayurveda. *Wrightia tinctoria* (Family: Apocynaceae; Malayalam: *Danthapala)* is a medium sized tree of the moist deciduous forests of some parts of India including the Western Ghats. The leaves of the tree ha5 been used in recent times under the Siddha system of medicine, as an effective remedy for a variety of skin ailments. *in vitro* studies were undertaken to develop a method of growing callus cultures and to induce plantlet formation.

2. MATERIALS AND METHODS

2.1. Plant materials

2.1.1. Bamboos

Seeds of *Bambusa bambos* and *Dendrocalamus hamiltonii* used in this study were stored in a dry condition in a refrigerator ($4^{o}C$) for two years. Seeds were germinated *in vitro* (as described below) and entire seedlings or nodes taken from the seedlings were used for culture.

Nodal explants of *B. bambos, B. bambos var.* gigantea, *B. vulgaris* and *T. oliverii* were collected from the secondary and tertiary branches of mature culms of plants growing in the field. The nades were used for culture after surface Sterilization within 24 h. of collection.

2.1.2. Rattans

Immature and mature fruits of the five species of rattans were collected from the climbers growing in the forest. Immature fruits were used for culture up to the third day after collection. Seeds were removed from the mature fruits by pounding them in a cloth hag to remove the pulp. Such seeds were stored in moist saw dust for up to a month until use. One year old seedlings obtained from seeds germinated in moist saw dust were also used in the experiments.

2.1.3. Medicinal plants

Plants growing in the botanical qorden of KFRI were the source of the material for culture. In *Kaempferia* spp. the rhizomes with fresh sprouts or dormant buds were used for initiation of cultures. In *Malaxis rheedii* the pseudobulbils were collected from plants immediately after flowering. In *Wrightia tinctoria,* nodal explants and leaves from mature trees growing in the KFRI campus at Peechi, were used as explants.

2.2, Surface sterilization

Explants were first prepared for culture by removal of dead tissue and cleaning by tap water containing a few drops of deterqent (Labolene) followed by 1-2 washes with distilled water to remove the detergent.

Mercuric chloride solution prepared in double distilled water was used for suface sterilization of all the plants used in this study. The concentrations and duration of the $HgCl_2$ treatment varied according to the nature of the plant material (Table

2.3. Glassware/ Culture containers:

Borosilicate qlassware was used in this study for preparation of culture media and for use as culture vessels. Test tubes used were nf the size 150 mm x 25 mm or LOO mm X 25 mm. Polycarbonate containers (Magenta Corporation, Chicago, USA) and polypropylene containers (Phytacon, Sigma Corp., St. Louis, USA) were also used for some of +he experiments and has been indicated in the appropriate place. Test tubes were plugged with nun-adsorbent cotton plugs. A11 empty glassware and water for surface sterilization of explants were autoclaved (1.5 kg $^{-1}$/cm^{-2}, 12l°C) for *20* min.

2.4. Culture media:

The mineral salts and vitamins according to Murashige and Skoog(1962) were used as the basal medium (Table 11.2) in all the experiments. Sucrose was added at 2 % w/v as the carbon source. All chemicals used in this study were of analytical grade (Merck or Qualigens), The medium was prepared using stock solutions of the mineral salts and vitamins (stored frozen) in distilled water. After addition of sucrose, necessary additions of growth regulators and other additives were made from stock solutions stored below So Z and the pH adjusted to 5-7 with 1 N NaOH or HCl. For preparation of solid medium, agar (Hi-Media, Bombay) was added at 0.15 % w/v and melted in a microwave oven before dispensing into the culture vessels. Aliquots of 20 ml were dispensed inta each test tube. Culture media were autoclaved at 1.5 kg $^{-1}$/cm $^{-2}$ at 121° C for 15 min. Table 11.3 gives the composition of different media used in this study.

Table 11.1 Surface sterilization of various explants using $HgCl_2$ solution

Explant	Concentration (%)	Time (min)
Bamboo		
Seeds	0.05	5
Nodal explants	0.10	5
Rattan		
Seeds	0.10	10
Seedlings	0.10	10
Kaempferia galanga		
Rhizomes	0.10	5
Wrightia tinctoria		
Seeds	0.10	5
Leaves	*0.05*	5

Table 11.2 Composition of Basal Medium (MS*)

Chemical	Concentration in mg/l
1. KNO_3	1900 00
2. NH_4NO_3	1650.00
3. $CaCl_2 \cdot 2\ H_2O$	440.00
4. $MgSO_4 \cdot 7\ H_2O$	370.00
5. KH_2PO_4	170.00
6. $MnSO_4 \cdot 4\ H_2O$	22.30
7. $ZnSO_4 \cdot 7\ H_2O$	8.60
8. H_3BO_3	6.20
9. KI	0.83
10. $CuSO4.5\ H_2O$	0.025
11, $Na2MoO4.2\ H_2O$	0.250
12. $COCl_2.6\ H_2O$	0.025
13. $FeSO_4 \cdot 7\ H_2O$	27.80
14. $Na2EDTA.2\ H_2O$	37.30

* – Mineral salts of Murashige and Skoog's Medium (1962)

Table 11.3 Composition of Culture Media

Basal Medium : MS + Sucrose 20 g/l *

Media Code	Supplements	
	Growth regulators (mg/l)	Other Supplements
Germination or Initiation		
G	-	—
IN	BA (0.5)	—
M Series (Multiplication media)		
M1	BAP (0.5, 1, 3,5)	—
M2	Kin (0.5, 1, 3,5)	—
M3	BAP + Kin (0.5,1,3,5 each)	—
A Series (Auxin containing media)		
A l	2,4-D (1,2, 3, 5)	—
A2	NAA (1,2, 3,5)	
Rooting Media		
R1	IBA (l, 2, 3, 5)	—
R2	-	AC 2 %

* - Agar (0.5 % w/v) added to obtain solid media wherever needed.
AC - Activated Charcoal

2.5. Culture techniques:

2.5.1, Bamboo:

Sterilized seeds, were first germinated on a solid medium to obtain sterile seedlings. Seedlings after 2-3 weeks of growth were used for further experiments for shoot multiplication. In one set of experiments the entire seedling with the seed was transferred to liquid medium (M series) in LOO ml conical flasks. These were either incubatea on a rotary shaker (70 r.p.m.) or stationary in light (10 umol m^{-l} s^{-1}). In another set of experiments, explants consisting of sinqle nodes were excised from the seedlings after 3-4 weeks and transferred to a solid multiplication medium (M series).

Nodal explants taken from mature culms were cultured on the M1, M2 and M3 media supplemented with Bavistin (0.2 % w/v) after a pretreatment with a solution containing Bavistin (0.2 % w/v) and 190 mg/l of strepto-penicillin for 60 min.

Subculturing was carried out at 4 week intervals by removi'ng the cluster of shoots and separating them into roughly equal sections consisting mostly of one long shoot with short axillary branches. During each subculture operation a proportion of the shoots with well developed root system were removed for transfer to soil.

Shoots with well developed roots were washed in tap water and transferred to a mixture of vermiculite-soil (1:3) in polythene bags. These were then kept in green house for hardening. Plants were shifted to polythene bags containing garden soil after 2 weeks and kept under partial shade in a nursery.

2.5.2. Rattans

The seeds were excised from fruits of all the species after sterilization. The sarcotesta from anterior end of the fruit was peeled out to expose the pulpy mesocarp. The hylar plug was then exposed by scraping with a scalpel and then removed. Using a lancet, the embrya was then teased out and transferred to the G or In medium for germination. Embryos were inoculated on to M series for shoot induction and multiplication and on A series to induce organogenesis or somatic embryagenesis. Calli and explants showing morphogenesis were shifted to G medium and light for inducing plantlet regeneration.

One-year-old seedlings of rattan germinated and grown on moist sawdust were also used as source of shoot meristem explants. The roots and dead tissue were completely removed and the shoot cut to isolate the collar region of about 2-3 cm long which included the shoat tip. In one set of experiments, the explant was cut longitudinally with a sharp scalpel from the shoot tip to about 3/4 th of the length of the explant. Collar explants were cultured on the multiplication media (M series).

Shoots were shifted to R1 medium and after a week to the R2 medium for rooting. Rooted shoots were transferred to a mixture of soil and vermiculite (3:1) and kept in a mist-chamber for hardening.

2.5.3, Medicinal plants

2.5.3.1. *Kaempferia galanga:*

Rhizomes were inoculated in test tubes on IN media containing 0.5 mg/l BAP to induce sprouting of the buds. The sprouted buds were separated from each other at the first subculture after four weeks and transferred to M1, M2 and M3 series for multiplication. Polypropylene containers and glass bottles were used for culture from the second subculture onwards. Subcultures were carried out at 5-week intervals when the leaves had elongated and filled up the culture vessel. At each subculture the clump of shoots were taken out and the leaves cut to a length of 2-3 cm to facilitate transfer. Single shoots were separated from each other by gently pulling them apart.

Rooted shoots were separated from each other and transferred to garden soil in polybaqs and kept in the shade for hardening. Hardened plants were planted in field at the onset of the rains. The traditional practice for cultivation of *K. galnnga* and similar crops like turmeric and ginger was followed. A raised bed of garden soil mixed with farmyard manure was prepared and plantlets dibbled at 30 cm X 30 cm spacing. Rhizomes of the previous years crop was planted as controls in adjacent beds'. Harvest of the rhizomes was done after eight months of growth, in February when all the leaves had dried completely.

2.5.3.2. Malaxis rheedii

Sterilized pseudobulbils were cut into sections with one node each and inoculated on all media in the M series. Subculture to fresh medium was carried out after sprouting of the axillary buds.

Shoots were shifted to the R1 medium for a week and then to G medium to induce rooting. Rooted plantlets were transfered to a mixture of vermiculite and brick-pieces and kept in a mist-chamber for hardening.

2.5.3.3. *Wrightia tinctoria*

Sterilised seeds were germinated on G medium. Leaf and internode explants taken from 4-week-old seedlings were inoculated on A series of media. Subcultures were carried out at 4-week intervals on fresh media for maintenance of callus. Transfer of the explants and callus to the IN and G media were carried out to induce morphogenesis and regeneration of plants.

3. RESULTS AND DISCUSSION

3.1. Bamboos:

Seeds of both the species germinated in the dark on G medium. The seedlings transferred to liquid medium M1, M2 and M3 along with the seed, formed multiple shoots from the base of the seedling (Fiq.III.1) in 2 weeks. The response in both the species was similar. Nodal explants (with at least two nodes per explant) taken from seedlings also developed multiple shoots from each node in 3-4 weeks. Dense clusters of short shoots formed after *6-7* weeks of culture on both liquid as well as solid media (Fiq.III.2). Although shoat multiplication was better on higher levels of cytnkinin (Table III.l), elongation of the shoots was poor. Thus media with lower levels of hormone was more suitable for obtaining plantlets. In liquid media and to a lesser extent on solid media, several long roots also developed from the lowest node on the longer shoots. On careful separation, 4-5 rooted shoots could be obtained from each culture which were transferred to soil in poly bags and hardened in the mist propagation chamber. For further multiplication, explants consisting of 1-3 nodes taken from the shoots were used. Liquid medium qave quicker and higher rates of shoot multiplication than solid medium (Table 111.2). No differences were observed between liquid cultures kept stationary ond those kept on a rotary shaker. The shoots with well developed roots on transfer to soil and hardening in a green house for 4 weeks gave up to *98* % survival. Plantlets developed new shoots in 6 weeks.

In one batch of cultures of *B.bambos* that had undergone more than 3 subcultures (and a total of 4 months in culture), in vitro flowering was observed. Clusters of erect spikelets occurred along with leaves. Spikelets were formed in solid as well as liquid media. Spikelets that were above the culture medium showed anthesis (Fig. 111.3). No seed set was however obtained and further veqetative growth did not resume as reported by Nadqauda et *al.* (1990).Flowering shoots could however be maintained over two subcultures of 4 weeks each after which the cultures did not survıve.

Two factors were associated with cultures that showed flowering. The shoots in these cultures were much shorter in length and qenerally showed a higher degree of senescence in the leaves. In two other reports of in vitro flowering (Nadgauda et al., 1990 and Rao and Rao, 1988), the medium contained a cytokinin and coconut milk. Results obtained in the present studies show that coconut milk is not essential thereby eliminating any factor of unknown composition in this complex additive, as responsible for induction of flowering. Chambers *et* al. (1991) also obtained in vitro flowering in *D. hamiltonii* on media devoid of coconut milk.

explants from mature culms gave high rates of bacterial and fungal contamination especially when taken during the rainy season. Pretreatment of the explants with antibiotics and culture

Table III.1 : Effect of cytokinins on shoot multiplication

Basal Medium : MS + Sucrose 20 g/l
Subculture Number : 3

Cytokinin	Concentration mq/l	Multiple shoats / culture	
		B. bambos	*D. hamiltonil*
BAP (M1)	0.5	2.0	2.2
	1.O	4.6	3.8
	3.0	8.5	7.2
	5.0	10.2	9.3
Kin (M2)	0.5	2.5	2.6
	1.0	3.7	3.4
	3.0	9.4	8.8
	5.0	12.3	11.2
BAP + Kin (M3)	0.5 + 0.5	3.0	3.4
	1.0 + 1.0	5.4	4.7
	3.0 + 3.0	8.3	7.6
	5.0 + *5.0*	12.2	12.7

Table 111-2: Effect of liquid and solid media on shoot multiplication in *B. bambos*

Basal Media : MS + BA (1 mq/l)+ Kin (1 mg/l)+ sucrose 20 g/l
Subculture Number : 3

Media	Shoots formed /explant
Liquid agitated	6.8
Liquid stationary	6.6
Solid	5.2

on a medium supplemented with Bavistin reduced the contamination rates to less than 50 %. Contaminants were mostly fungal and typically appeared on the explant after a week of culturing. The presence of the fungal mycelium only on the cut surface of the explant indicates that the contaminant is endogenous in origin.

On M3 media with 1-3 mg/l of Kin and BAP supplemented with Bavistin (0.2 % w/v) the axillary buds sprouted and elongated one week after culturing. At each node 2-4 shoots are formed. Subculture to fresh media after 4 weeks did not elicit continued growth or multiple shoot formation. Transfer of explants to liquid medium was also not effective for maintenance of shoot cultures. Transfer of the explants to R1 medium to induce rooting was unsuccessful.

A higher rate of multiplication than by conventional means is still possible if the sprouted buds could be rooted while still attached with the original explant. Nadgir *et al.* (1984) reported multiple shoot formation and rooting at a low frequency in nodal explants collected from mature culms of *B. bambos (B. bambos), B. vulgaris* and *D. strictus.* The difficulty in obtaining results with mature bamboo explants is reflected in the absence of any other report even though a large number of laboratories are carrying out work on microprooagation of bamboos. A judicious selection of antibiotics and collection of explants during the appropriate season is probably the strategy to be adopted to solving the problem in Kerala.

3-2. Rattans

Embryos cultured on the G and IN media enlarged and turned green after 4 weeks. The plumule and roots continued to develop as in the germinating seed if the embryo is shifted to fresh media every 4 weeks. Embryos cultured on M series expanded in size in the first two week5 and developed into a plantlet. When the plumule is split longitudinally with a scalpel and cultured fresh shoot initials developed at the collar region in 8 weeks in all the three species. In explants transferred to a liquid medium at this stage the shoot development was quicker (Fiq.111. 4). Shoot initials continued developing and formed a cluster of shoots in this medium. When the clusters were separated into groups of 2-4 and subcultured to fresh medium, new shoot initials were induced to develop at the base. Multiple shoot formation in C. *thwaitesii* and C. *hookerianus* was highest on media containing 3 mg/l Kin and 0.5 mg/l BAP while in C. *pseudutenius* media with 3 mg/l each of Kin and BAP gave the best results (Table 111.3). Padmanabhar and Illangovan (1994) have reported similar results in cultured embryos of C. *gamblei* that were cut in the collar region before culture. The disturbance of the apical meristem and a release of the apical dominance may be the reason for the induction of multiple shoots in these rattans.

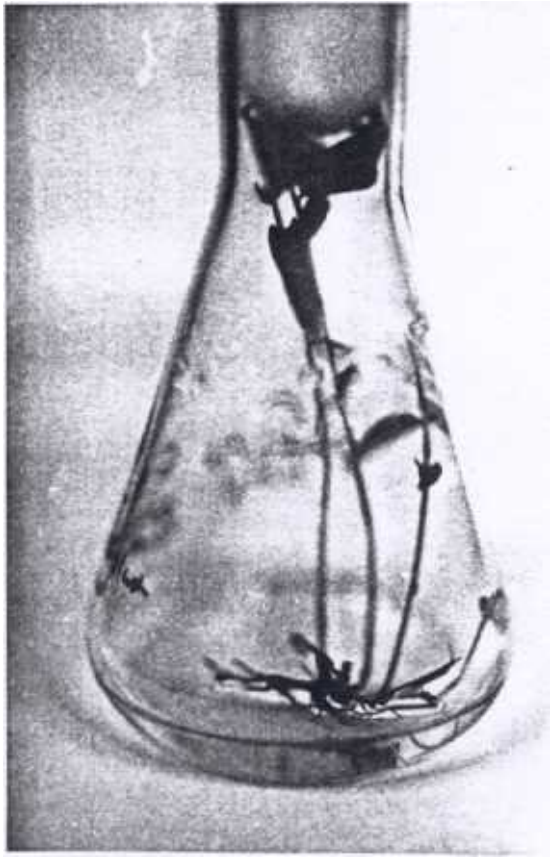

Fig.III.1 : Multiple shoot formation at base of seedling of *B. bambos* in liquid medium.

Fig.III.2: Cluster of multiple shoots *D. hamiltonii* growing on solid medium.

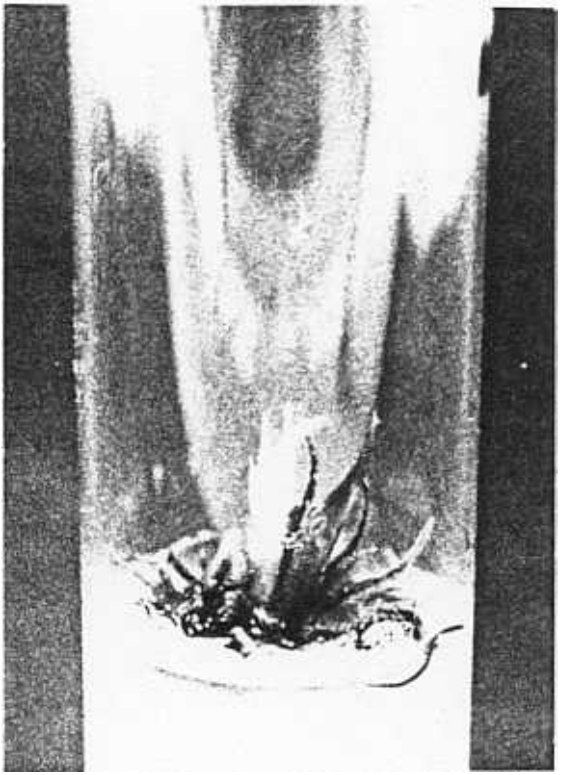

Fig. III.3 : Shoot culture of *Bambusa bambos* showing *in vitro* flowering.

Table 111.3: Effect of cytokinins on multiple shoot formation in rattans

Basal Medium : MS + 20 q/l sucrose
Subculture No. : 3

Cytokinin	Conc. mg/l	Multiple shoots / cu1ture C.tw.	C.ps.	C.hk.
BAP	1.0	3.2	4.2	4.2
	3.0	5.6	5.6	4.8
	5.0	5.4	4.8	4.8
Kin	1.0	5.0	5.8	5.2
	3.0	5.6	6.6	5.8
	5.0	5.6	6.4	6.0
BAP + Kin	0.5 + 3.0	6.6	7.2	6.0
	1.0 + 3.0	7.0	7.4	6.6
	3.0 + 0.5	6.0	6.4	5.8
	3.0 + 1.0	6.0	6.8	6.0
	3.0 + 3.0	6.6	7.8	6.2

C.tw.- C. *thwaitesii;* C.ps.- *C. pseudotenuis;* C.hk.- *C.*

Root formation occured in the multiplication medium on some of the shoots in the cluster in all the three species. About 30 % of the shoots shifted to R1 medium for a week and then to R2 medium developed roots in one week. Plantlets shifted to a soil: vermiculite (3 : 1) mixture and hardened in a mist propagation chamber for 4-8 weeks;, survived and grew well when transferred to soil in earthen pots. In *C. rotang* Padmanabhan and Ilangovan (1989) had obtained rooting of *in vitro* raised seedlings by transfer to a sugar free medium.

In C. thwaitesii clusters of about 3-6 shoots with more or less the same length and thickness and a few shoot initials, were formed. When entire clusters were treated for rooting without separation of the individual shoots, the clumps of shoots with roots could be transferred to soil. This raises the possibility of obtaining early clustering in rattan species that normally form clusters only in 3-4 years. Further observation of the plantlets will be required to confirm the pattern of growth of the shoots. The potential of induced early clustering in rattans in plantations are tremendous since it could improve stocking capacity as well as permit multiple harvests. An additional possibility is of inducing rattan species that are normally non-clustering to produce clumps in culture and thereby in field.

Embryos cultured on A series and incubated in dark, showed irrequlars swellings on the surface after 2 weeks. On media supplemented with 2,4-D, a soft brown callus developed on the surface of embryos after 3-4 weeks of culture. A hard callus is formed at the higher concentrations of NAA (3 and 5 mg/l). In cultures derived from embryos of C. hookerianus, an embryogenic tissue develops after the third subculture. The cultures had the appearance of a clump of embryos. When calli were shifted to hormone free medium (G) after 2 weeks of culture to induce morphogenesis, development of the somatic embryos occurred at a low frequency and without any synchroniztion (Fig. 111. 5). Embryos could be separated from each other during subculture. In embryogenic cultures shifted to a liquid M1 or M2 medium (with 0.5 mall BAP or 3 mg/l Kin), conversion of embryos was possible in 2-3 weeks. Germinating embryos (emblings) were shifted to separate tubes to allow further development of shoot and root (Fig. 111.5). After 5 weeks of culture the emblings were removed from culture, washed free of media and transferred to a sterilized mixture of soil and vermiculite (3:l) kept in a mist Propagation chamber for months to permit hardening. Plants were then transferred out to partial shade.

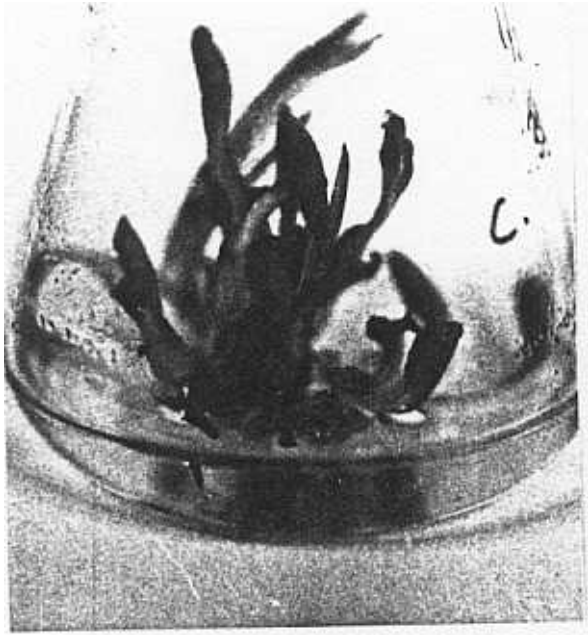

Fig. III.4 : Multiple shoots of *Calamus thwaitesii* in liquid culture.

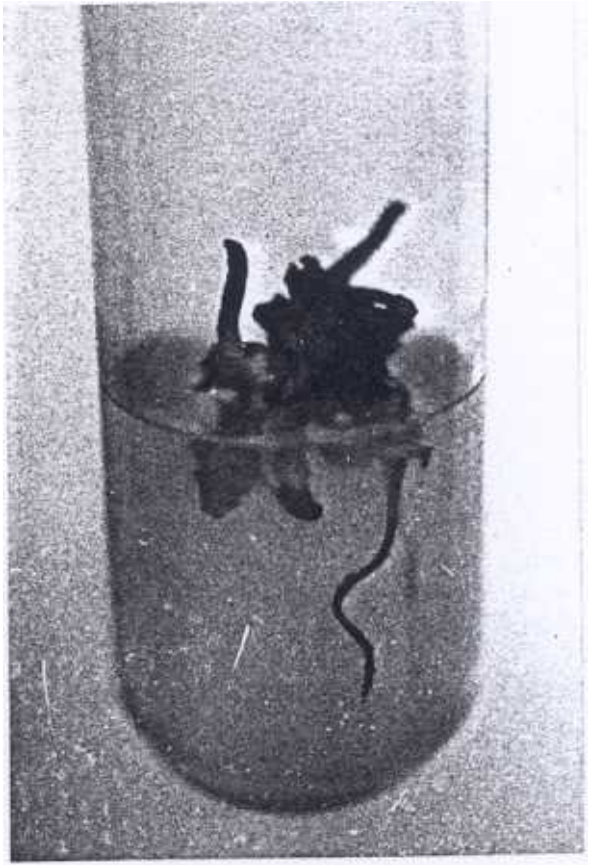

Fig.III.5 : Somatic embryos of *Calamus hookerianus* with shoot and root development.

3.3. Medicinal Plants:

3.3.1. *Kaempferia galanga :*

Rhizome sections cultured on IN formed shoots from the dormant buds in 2 weeks. From the first subculture onwards multiple shoot formation was evident in all the cultures. Multiple shoot formation at each subculture was 5-8 fold in K. galanga (Fig. III.6) Since the shoots elongated rapidly to fill up the test tubes, cultures (from the second subculture) were done in jlass bottles or oolypropylene containers which permitted expansion of the leaves (Fig. 111.7). Root formation was observed to occur along with shoot development (Fiq.III.8). It was thus possible to separate complete plantlets at the end of each subculture.

Plantlets transferred to garden soil and placed in shade for hardening survived without any need for like misting or use of plastic tent. Wilting of leave.;. commonly observed in microprnpagated plants exposed to ambient conditions, was not observed if plants were watered daily. After about 10 weeks of hardening, plantlets could .be transferred to earthen pots (Fig.III.9).

Table 111.4 qives a camparision of the microprapagated plantlets with the control plants derived from rhizomes. Plantlets of K. galanga had thin and elongated leaves with a petiole like portion. Leaves in the micropropagated plants were also aligned almost vertical to the plant axis (Fig.III.9). In contrast, the leaves in normal plants (those developing from rhizomes) were rouna, sessile and aligned horizontal to the olant axis.

Preliminary observations carried out on the plantlets transferred to the soil show some differences in the growth pattern of micropropagated plants as compared with the plants grown from conventional propagules viz. rhizome. Leaves of micropropagated plants of K. galanga wilt and die especially if exposed to direct sunlight and rain. However new leaves are formed within one week. These leaves have relatively normal morphology viz. shorter petioles and wider lamina. Micropropagated plants still have larger number of leaves than control plants. This is directly related to the number of rhizome buds developing per plant (see below).

At the time of transfer of plantlets from polybags to pots, a thickening of the basal portion of the plants was evident besides the oresence of initials of small shoots developing from the axillary buds. At the end of the growing season viz. February, the rhizomes from the micropropagated as well as the controls were harvested and weiqhed after cleaning and removing the roots and leaves. The number of buds formed per plantlet was also counted. This gives an estimation of the total number of plantlets that could be produced from the rhizomes in the next growing season. The data on rhizomes obtained from the plants qrown for one season viz. eight months (July to February) is given in Table III.4.

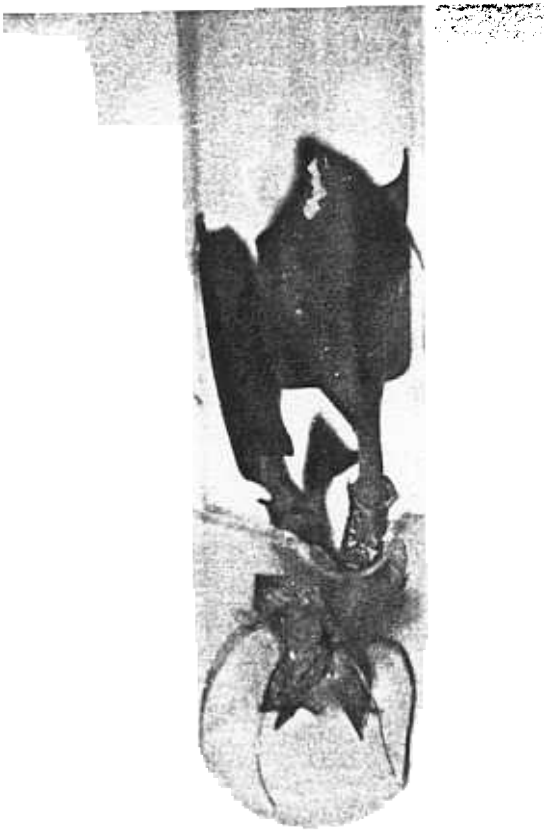

Fig. III.6 : Multiple shoot formation in rhizome explants of *Kaempferia galanga*.

Fig. III.7 : Shoot cultures of *K. galanga* grown in glass bottle.

Fig. III. 8 : Rooted shoot of *Kaempferia galanga*

Fig. III.9 : Plantlet of *K. galanga* in soil after hardening.

Table 111.4 : Comparison of micropropagated plantlets with vegetatively propagated plants of K. galanga

	Micropropagated plantlets	Control plants (Vegetatively propagated)
Leaf *		
Length	13.70	13.35
Width	2.16	5.85
Rhizomes **		
Number of buds	26.90	14.40
Fresh weight	40.80	42.18

* Average of 5 plants; 5 leaves per plant (only *ex vitro* leaves).

** After one growth season (Average of 20 plants;)

While the total biomass of rhizomes produced by the micropropagated *K. galanga* plantlets as well a5 the controls is comparable, the rhizome in the former consists of a number of small and slender branches; whereas the normal morpholoqy is seen in the control rhizomes. Since each branch of the rhizome produces a shoot, the total number of off shoots per clump is greater in the micropropagated plants.

The rhizome af *K. galanga* is generally marketed as dried thin slices. Thus, use of micropropagated plants as propagules for planting is expected to result in a poorer quality of the rhizome. This observation has to be viewed only in the context where planting was done with no external input of fertilizer. It can be attributed to the absence of a rhizome and smaller veqetative parts of the micropropagated plant. However one advantage of using micropropagated plants was that multiplication of shoots at the end of the qrowing season was at least 3-4 times that of the control plant. The immediate application that micropropagation. lends itself to, is large-scale production of planting material when there is a shortage of rhizomes.

3.3.2. *Malaxis rheedii*

In the nodal explants cultured on the M1, M2 and M3 series of media sprouting of the axillary bud was observed from the 3rd week (Fiq.III.10). Further growth of the buds was however slow. Nu response other than elongation of the buds occured in the first two subcultures. Multiple shoot (bud) formation was observed after the third subculture on M2 and M3 media. Cultures appeared a5 masses of numerous shoot initials (Fig.II1.11). Highest multiplication rates were obtained on media with *5* mg/l BAP and 3 mg/l Kin + 0.5 mg/l BAP, but elongation of the shoot initials did not occur.

Rooting of shoots wa5 obtained in only a few shoots shifted to R1 media. Rooted shoots transferred to a mixture containing brick pieces and vermiculite showed poor surviva1.

3.3.3. *Wrightia tinctoria*

Callus formation from leaf and internodes were obtained on A1 and A2 at all the concentrations. A white soft and friable callus was formed in a week of culture. On subculture of the callus on the respective media maintenance and growth of the callus was possible up to 3 subcultures. To induce morphogenesis the explants and calli were transferred from the auxin containing media to IN or G media which were auxin free. Calli continued to grow slowly for 2-3 weeks but eventually turned brown and senescent after 5 weeks. No indication of morphogenesis was observed except for root formation in a few cultures.

The lack of morphogenetic competence in callus cultures can be attributed to several factors. In an explant like the leaf or internode which consists of a several types of tissues, the

morphogenetically competent cells need not necessarily be the ones which proliferate to form a callus under a given set of conditions especially media composition. It may also happen that the conditions in which the callus was grown, especially the presence of auxins, does not permit morphogenesis even after the conditions were changed (in this case the transfer to hormone free medium). This may be due to a change in the endogenous level of growth hormones or a direct effect of media compomponents on genetic expression.

Fig. 111.10 : Sprouting of the axillary bud in nodal explant of *Malaxis rheedii.*

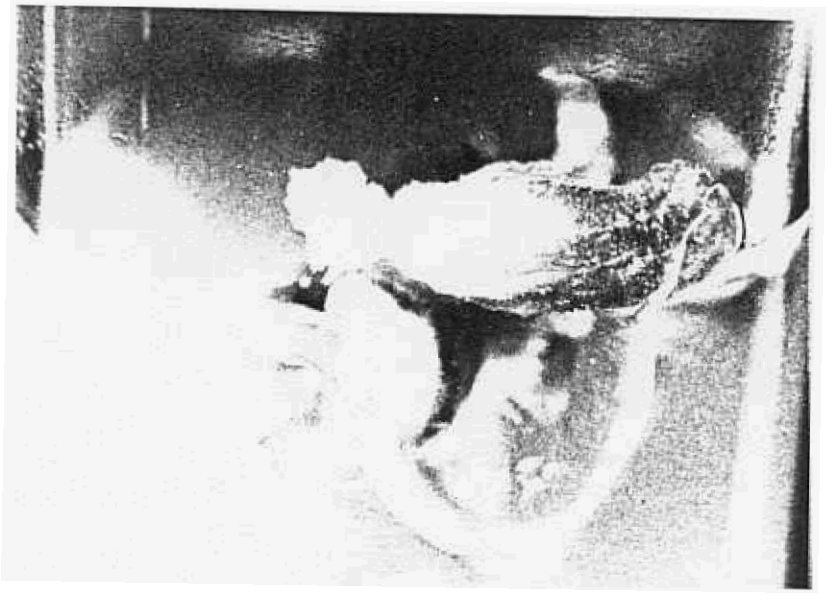

Fig. 111.11 : Multiple shoot formation in *M. rheedii*

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