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PROGRESS REPORT NO. 1

TISSUE CULTURE OF TEAK (Tectona grandis)

A RESEARCH PROJECT

USAID/PSTC PROGRAMME

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SUBMITTED BY

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## PROJECT PROFILE

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## INTRODUCTION

Tissue culture (in vitro culture) in forestry is one of the most interesting subjects for research and development during the last two decades. Several attempts have been made to mass propagate forest tree species by means of this technique. A number of studies on tissue culture of tree species were presented and discussed in the International Union of Forest Research Organization (IUFRO) meeting in Paris in 1981 (IUFRO, 1981). The current status of tissue culture in forestry has also been reviewed and edited in 1982 by Bonga and Durzan. Despite this sizeable literature in this field it is still in doubt whether the techniques developed under experimental scales can be applied and/or practised for a large-scale nursery production conditions.

As for teak, pioneer studies on teak tissue culture were conducted in India (Gupta et al, 1980 and Masarenhas et al, 1981) and showed that teak can be propagated successfully by using the tissue culture technique. In these studies, explants derived from both buds and/or shoot-tips of mature trees and sterile seedlings were cultured in vitro on the MS (Murashige and Skoog, 1962) agar culture medium supplied with cytokinins BAP and Kinetin. About 30 days of culturing, the cultured explants regenerated shootlets and they were, thereafter, cut into 5-10 mm. segments to produce more shootlets. These shootlets were separated individually for in vitro rooting. Through this technique, it was calculated that

about 500 and 3,000 plantlets can be obtained from the cultures of a single bud and/or shoot-tip of mature tree and a single sterile seedling, respectively, within a year.

Similarly, a series of studies on teak tissue culture was conducted in Thailand during a period of 1983-1985 by the joint research team from Chiang Mai University and the Royal Forest Department (Apavatjirut, Kaosa-ard and Sombun, 1985). In these studies, explants derived from both buds and/or shoot-tips of mature trees and from sterile seedlings were cultured in vitro on the culture medium suitable for teak shoot multiplication. The results of these showed that (a) such in vitro culture technique can be used successfully for teak propagation and (b) there is a high potential to improve the developed techniques for future mass nursery production.

Based on the studies conducted both in India and in Thailand, it is clearly indicated that teak can be propagated by using the tissue culture technique and the cultured explants can be both buds and/or shoot-tips of mature trees and sterile seedlings. The developed technique in those studies are, however, needed to be improved for individual clone and also for suitable commercial operation and cost effective if it is going to be used for a large-scale nursery production.

## PROJECT OBJECTIVES

The general objectives of this research project are as follows:

- 2.1 To develop in vitro culture techniques for mass propagation of teak explants from sterile seedlings and from shoot-tips of mature trees.
- 2.2 To observe field performance in terms of survival rate, growth rate and variation of plants derived from the developed and/or improved techniques in 2.1.
- 2.3 To evaluate the cost of production of teak planting stock from both the in vitro culture and conventional techniques.
- 2.4 To develop suitable medium and conditions for regeneration of shootlets and/or plantlets from callus culture.
- 2.5 To establish techniques of anther cultures for future studies on genetics and improvement programme of the species.

## WORK IN PROGRESS

During the first 6-month period (May-October 1986) of this research project, the research and development activities on teak tissue culture were divided into 3 main parts. The first part was the improvement of the existing techniques developed by the research team. The second part was the development of the anther culture and the callus culture of the species. The last

part was the improvement of laboratory and nursery facilities for the research project. The work progress for each part during this first 6-month period are presented as follows:

3.1 PART 1 : IN VITRO CULTURES OF STERILE SEEDLING AND SHOOT-TIP EXPLANTS

Plant Materials : Teak fruit (seed) and shoot-tips of mature trees, used in this part of studies were those of genetically improved materials derived from the Teak Improvement Centre (TIC), Ngao, Lampang.

Laboratory : The laboratory and in vivo transplanting studies were conducted at the Faculty of Agriculture, Chiang Mai University.

3.1.1 STUDY 1.1 : Mass in vitro Propagation by Culturing Sterile Seedling Explants

3.1.1.1 Objectives

The specific objective of this study during this report period is to prepare a large number of plantlets for future studies on :

- (a) the improvement of in vitro culture technique for shootlet multiplication;
- (b) the improvement of rooting techniques of shootlets;
- (c) the stability of the plantlets obtained from (a);
- (d) the cost estimation of mass planting stock production.

#### 3.1.1.2 Methods

Teak fruits from the TIC seed orchard and seed production areas were brought in for the study in June 1986.

In the laboratory part, the seeds (the white seeds) were excised from the fruits (the dry drupe) by cutting the fruits with a sharp knife. The excised seeds were surface sterilized by shaking in 10 % (v/v) Chlorox solution for 10 minutes and thereafter they were rinsed in sterile distilled water for three times. The sterilized seeds were transferred singly onto White (1963) agar culture medium (without growth hormones) in a test tube under aseptic conditions and kept at about 28 C under continuous light for germination.

Forty five days after germination, the sterile seedlings were removed and dissected under aseptic conditions into small sections or explants for culturing. About 4 sections of explants (one shoot-tip and three stem nodal sections) are, generally,

obtained from a seedling. The explants were then transferred to culture onto the agar medium developed specially for the teak shootlet multiplication in 250 ml-erlenmayer flasks or bottles (jam-jars). The cultures were kept at about 28<sup>o</sup> C and supplied with fluorescent light during the 16-hr night period. During the 8-hour-day period, the light was supplied from the glass-windows of the culture room. The compositions of the developed culture medium are as follows:

Macronutrients			Schenk and Hildebrandt (SH) (1972)
Micronutrients			Murashige and Skoog (MS) (1962)
IBA	0.3	mg/l	Organic additives (MS, 1962)
BAP	1.0	mg/l	
Sucrose	30.0	g/l	Nicotinic acid 0.25 mg/l
Agar	10.0	g/l	Pyridoxine. HCl 0.25 mg/l
			Thiamine. HCl 0.25 mg/l

Forty five days after culturing, the shootlets (about 3-5 cm in length) which were developed from shoot-tips and/or axillary buds of the cultured explants were subcultured for re-multiplication. To subculture, the shootlets (about 1-5 shootlets/explant) were removed from flasks/bottles and dissected into new or the second-cycle explants. Up to 4 explants can be obtained from a shootlet. The new explants were then transferred to culture onto the culture medium as given above. These subculture processes were repeated at 45 day intervals throughout the report period of May-October 1986 for mass shootlet production. It should be mentioned that a part of these shootlets derived from initial cultures maintaining as stock cultures before this research project started in May.

### 3.1.1.3 Results

During the first report period (May-October 1986), a large number of teak shootlets were produced by means of in vitro culture of sterile seedlings explants (Table 1), aiming to produce sufficient materials (50,000 shootlets) for future studies as given in section 3.1.1.1.

Although the present technique of in vitro culture of teak seedling explants was used satisfactorily in this study especially in terms of shootlet multiplication rate, there were, however, two main problems encountered in this multiplication.

Firstly, there was a large variation in size (1-5 cm in length) of the regenerated shootlets (Plate 1) at the subculturing and/or rooting time (about 45 days after culturing). As a result, the proportion between the bigger shootlets which are most suitable for subculturing and/or rooting (about >2.5 cm in length) and the smaller shootlets was the difference in shoot size as presented in this study was perhaps due mainly to : (a) the difference in regeneration rate of shootlets within the cultured explants and/or (b) the growth competition of the regenerated shootlets, i.e. the faster regenerated shootlets dominated the later regenerated ones in the same explants.

Secondly, the space in culture room is not sufficient for mass (>10,000 shootlets per cycle) propagation. The culture

bottles in this study were densely placed (170 bottle per 0.85 x 1.5 m-shelf) on the 4-layer shelves (Plate 2). As a result, there was a degradation in growth and health of the newly regenerated shootlets. This was mainly due to an insufficient light supplied to the cultures. It was observed that, the light intensity in the middle of the shelf-stands was only 50 lux during the day time (without artificial light supplied) and about 150 lux during the night time (supplied with inforescent light for 16 hours). Moreover, the cultured explants in the bottles were also shaded by the metal lids of the bottles (Plate 3). Since teak is known as a light demanding for growth and development species, the culture space and/or light problem in this reseach project is seriously to be tackled.

Table 1 Number of shootlets and cultured explants produced from sterile seedling explants in the laboratoy in October 1986.

Number of flask/bottles with subcultured nodal explants	125
Number of flasks/bottles with growing shootlets	1,790
Number of growing shootlets (approximately)	35,800

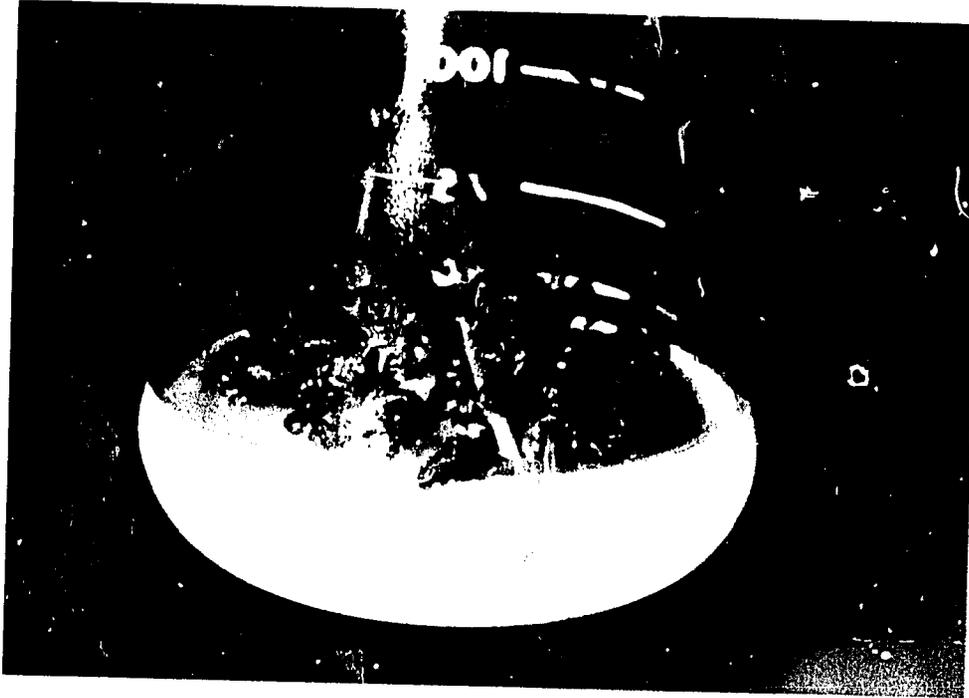


Plate 1      Shootlet regeneration from culture explants.  
(Showing variation in number and growth of shootlets)



Plate 2      Culture bottles in the culture room.  
(Showing the limited of culture space and light)



Plate 3 Culture bottles (jam jars) used in the study.  
(Showing the metal lid which causes shading to the shootlets)

#### 3.1.1.4 Conclusion/Remark

3.1.1.4.1 Shootlets from sterile seedling explants had been satisfactorily prepared in a large quantity for future study.

3.1.1.4.2 If the variation in growth and development of shootlets regenerated from the same culture explant (nodal section) can be reduced, the rate of shootlet multiplication and production will be even faster.

### 3.1.1.5 Future Study for the Following Period

3.1.1.5.1 To continue multiplying the shootlets for employing in relevant studies.

3.1.1.5.2 To conduct research as stated in section 6.1.1-6.1.5 of the project proposal by using the regenerated shootlets obtained from this present study.

3.1.1.5.3 To conduct research as stated in sections 6.1.9 and 6.1.10 in the project proposal.

## 3.1.2 STUDY 1.2 : Mass in vitro Propagation by Culturing Shoot-tips of Mature Trees

### 3.1.2.1 Objectives

The specific objectives of this study are :

- (a) To improve the developed technique for clonal mass propagation of teak by means of in vitro culture of shoot-tips from mature trees.

- (b) To study the effects of clone and season of shoot-tip collection on shoot regenerating potential of the cultured explants (shoot-tips).
- (c) To study the effects of in vitro culture on growth and development of plants obtained.
- (d) To estimate the cost of plantlet production.

### 3.1.2.2 Methods

Teak shoots of mature trees (>20 year-old trees) from the TIC clone bank in Lampang province (about 180 km from the laboratory) were used in this study. At each culturing time, the small lateral branches (about 5-10 cm in length) of trees in the clone bank were detached. The detached branches containing buds then sprayed with fungicide (Benlate), kept in an ice box and transported to the laboratory.

In the laboratory, the shoot-tips were removed and washed in running tap water. To prepare explants for culturing, the base and the sprouting-leaf parts (in active growing period) of the shoot-tips were first cut off leaving only the tip parts of size approximately 1.0 cm in length. As for buds in the dormant period (November-February), their bud scales (Plate 4) and/or the young leaves of the tips were then carefully trimmed off with a scalpel, and washed in running tap-water. The prepared shoot-tip explants were then surface sterilized by shaking in the 10-20% (v/v) Chlorox solution unless otherwise stated for 10-20 minutes. The Chlorox shaken explants were rinsed for three times with sterile distilled water. The outer parts of the surface

sterilized shoot-tip explants were trimmed off again under aseptic/microscopic conditions into about 0.1-0.3 cm in size. They were then transferred singly to test tubes containing agar culture medium. The composition of the medium developed for teak shoot multiplication was the same as that given previously (Section 3.1.1.2).

Within three weeks after culturing, the shoot-tips of the responsive cultured explants (within and among clones) started regenerating shootlets, whereas those non-responsive cultured explants started forming callus (Plate 5).

Forty five days after culturing, the regenerated shootlets (about 4-5 cm in length) were removed and dissected into small nodal sections, i.e. the shoot-tip and the stem nodal parts. These sections were used as the subcultured explants for shoot multiplication. The prepared explants were then transferred into flasks and bottles (jam jars) containing the same culture medium and under the same culture conditions as described previously.

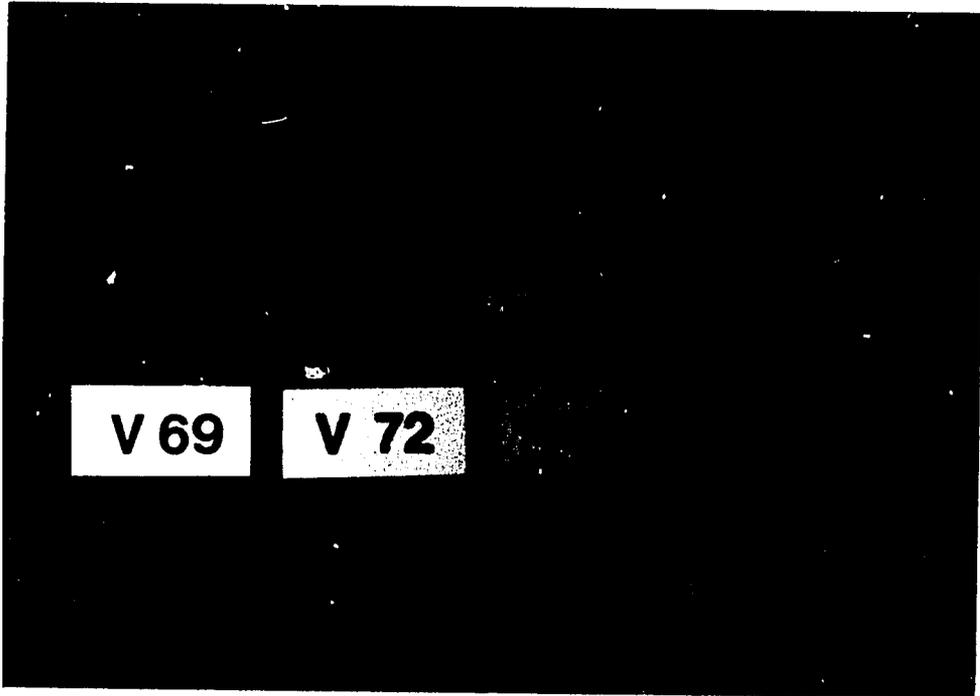


Plate 4      Buds from mature trees in a dormant period.

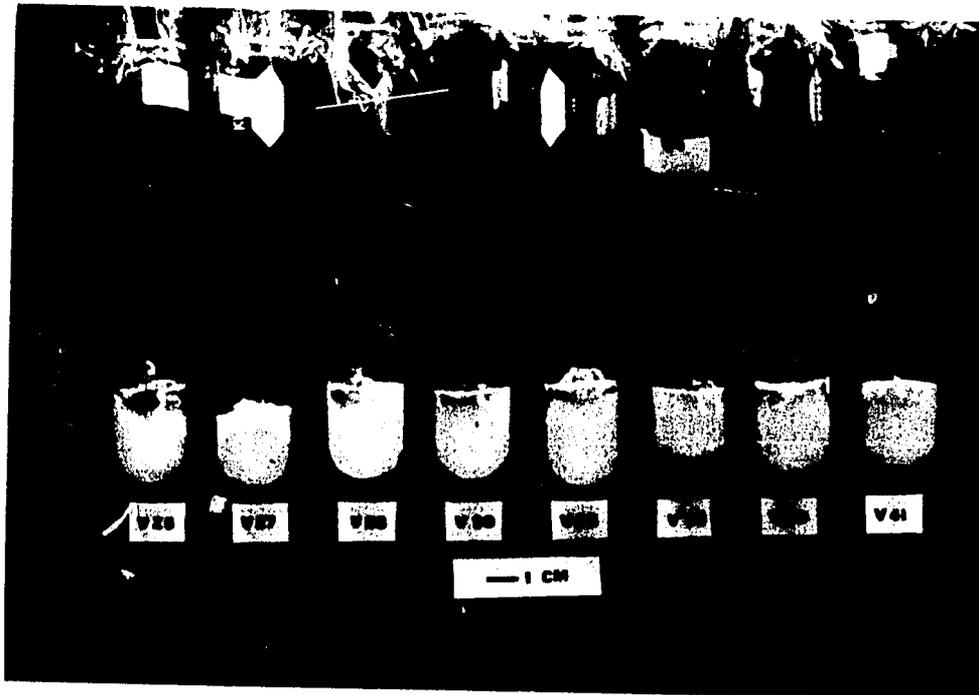


Plate 5      Clonal variation in shoot regeneration of in vitro cultured shoot-tips

To study the effects of clones and season or time for shoot-tip collection on shoot regenerating potential after in vitro cultures, five different clones, i.e. clone numbers V7, V36, V37, V39, V93 and V 181, were selected and used in this study. The shoot-tips of these selected clones were taken and cultured at monthly intervals from October 1986 throughout the study period (October 86 - September 87). The characteristics of shoot-tips at each collection time in relation to the rate of contamination and the responsiveness to the culture of the cultured explants were observed for each clone.

### 3.1.2.3 Results and Discussion

All of the 149 shoot-tips from 21 clones cultured during 6-13 August, 1986 were unexpectedly contaminated. In the same month, during 21-24, when another lot of 232 shoot tips from 17 clones were cultured, not all were contaminated, the contamination rate was reduced to 41.7-86.6% according to the clones (Table 2).

Table 2 Number of clones and cultured shoot-tips from mature trees during the reporting period.

Date of culturing	Clone no.	No. cultures	Contamination No.		Remarks
6-13 August 86	V 161	8	8	100	1. The shoot-tips from 21 clones were cultured during the period of 6-13 August 1986.
	V 163	8	8	100	
	V 164	8	8	100	
	V 166	9	9	100	
	V 167	7	7	100	
	V 174	6	6	100	
	V 176	8	8	100	
	V 177	7	7	100	
	V 179	6	6	100	
	V 184	3	3	100	
	V 186	7	7	100	2. The shoot-tips were sterilized by using 10% Chlorox for 10 minutes.
	V 188	8	8	100	
	V 196	6	6	100	
	V 201	5	5	100	
	V 209	6	6	100	
	V 217	7	7	100	
	V 220	8	8	100	
	V 222	8	8	100	
	V 226	8	8	100	
	V 228	8	8	100	
V 240	8	8	100	3. There were small brownish dots in the tissue of the shoot-tip explants before culturing during this culture period.	
21-24 August 1986	V 25	11	8	72.7	All the shoot-tips of 17 clones were sterilized by using 10% Chlorox for 10 mins.
	V 27	13	6	46.1	
	V 28	12	9	75.0	
	V 30	15	8	53.3	
	V 35	15	13	86.6	
	V 38	11	8	72.7	
	V 40	15	12	80.0	
	V 41	14	11	78.6	
	V 43	14	8	57.1	
	V 45	14	12	85.7	
	V 46	14	10	71.4	
	V 49	14	10	71.4	
	V 69	15	7	46.6	
	V 71	13	8	61.5	
	V 72	12	5	41.7	
	V 73	14	12	85.7	
	V 78	16	10	62.5	

Table 2 (cont.)

Date of culturing	Clone no.	No. cultures	Contamination No.	%	Remarks
3 September 1986	V 3	19	19	100	1. Shoot-tips of clones V3, V4, V7, V26 and V27 were first sterilized by using 10% Chlorox for 10 mins. Their outer leaf scales were then removed and they were resterilized by using 5% Chlorox for 10 min.  2. Shoot-tips of the remaining clones were presterilized by soaking in fungicide (Benlate) for 1 day. They were then sterilized by using 20% Chlorox, 10 mins.
	V 4	11	11	100	
	V 7	11	11	100	
	V 26	12	12	100	
	V 27	14	14	100	
	V123	11	11	100	
	V124	11	11	100	
	V126	12	12	100	
	V133	9	9	100	
	V135	11	11	100	
	V138	10	10	100	
	V140	8	8	100	
	V159	8	8	100	
	6 October 1986	V 7	12	12	
V 36		24	24	100	
V 37		21	21	100	
V 39		23	23	100	
V 93		41	41	100	
V164		11	11	100	
V170		12	12	100	
V174		11	11	100	
V178		10	10	100	
V181		25	25	100	
V184		11	11	100	
V188		12	12	100	
V190		11	11	100	
V197		10	10	100	
V199	10	10	100		

In September, 3 the sterilizing technique (10% Chlorox, 10 mins) used for both lots of materials in August was improved by surface sterilizing the buds, after removing the outermost leaf pair (still closed and non sprouted), they were first sterilized in 10% chlorox (v/v), rinsed in sterile distilled water once and agitated again, after removing another leaf pair, in 5% chlorox (v/v) for 10 mins and rinsed 3 times in sterile distilled water. The results showed that with the improved techniques, all of 67 buds from 5 clones were still contaminated.

Another effort was conducted by presterilized the 80 buds of 8 clones in a solution of fungicide (Benlate) for 30 mins in the clone bank before transported to CMU. In the laboratory, the pre-treated buds, with one leaf pair and/or bud scales removed were sterilized in a solution of 20% Chlorox (increased concentration) for 10 mins and rinsed 3 times in sterile distilled water.

All of the cultured buds were still contaminated as those cultured on September 3, and the same as another trial of 15 clones cultured on October 6 (Table 2).

The very high rate of contamination is normally not common in shoot growth flushing period. From our previous experience surface sterilizing sprouted buds in May by 10% Chlorox (v/v) for 10 mins is sufficient, and our previous work only cultured the buds in May.

However, this serious problem of contamination in shoot tip culture during this flowing/fruiting period (August-September) was tried to be solved again in the following month (Table 3). Only 5 clones were used in this study. The results of the 3 treatments tested i.e. Mercuric chloride 0.1% (w/v) for 20 mins, 20% chlorox for 20 mins, and dipping in 70% ethylalcohol for 45 sec. and followed by sterilizing in 20% chlorox solution for 10 mins.

In general, less contamination percentage was obtained from those buds sterilized with mercuric chloride than that occurred in the cultured buds sterilized by other two sterilizing procedures, whereas dipping alcohol solution prior to sterilization in 20 % chlorox was ineffective.

The extremely high contamination percentage encountered during the fruit setting period (August-September), which is uncommon during the shoot/bud sprouting and flushing period (April-June), is due primarily to :

- (1) the high rate of activities of all associate microorganisms in both outer and inner buds during during this period (the onset of dormant bud period);
- (2) the high rate of activities of insects (bud borers) causing brown spots in the buds and contamination from inside of the cultured buds were frequently resulted;

(3) the hard bud scales causing technical problem in preparation of culture explants.

#### 3.1.2.4 Future Study for the Following Period

3.1.2.4.1 All the survived superiod clonal bud cultures will be subcultured to obtain more shootlets sufficient for the following work : -

3.1.2.4.1.1 A study on comparison of the cultured clones in order to gain informations on their growth and multiplication capacity of each clone.

3.1.2.4.1.2 The clonal shootlets obtained will be multiplied for transferring and rooting in vivo. Thereafter, the rooted transferred plantlets will be used in stock stump production at Teak Improvement Centre, Ngao, Lampang.

3.1.2.4.2 More buds of other superior clones from the germplasm bank of Teak Improvement Centre will be cultured from a study on clonal comparison (as mentioned in 3.1.2.4.1.1 above) and multiplied, preparing for the same purpose as in 3.1.2.4.1.2.

Table 3 Effects of sterilization techniques on contamination percentage of cultured explants (shoot-tips).

Date of Culturing	Clone No.	Methods of Sterilization	No. Cultured Shoot-tips	Contamination	
				No.	%
6-8 October 1986	36	Mercuric chloride 0.1%	7	3	42.8
	36	Chlorox 20%, 20 mins	10	8	80.0
	36	Dipped in 70% ethyl alcohol for 45 secs, then sterilized in 20% chlorox, 10 mins.	7	7	100.0
6-8 October 1986	37	Mercuric chloride 0.1%	-	-	-
	37	Chlorox 20%, 20 mins	10	6	60.0
	37	Dipped in 70% ethyl alcohol for 45 secs, then sterilized in 20% chlorox, 10 mins.	11	8	72.7
6-8 October 1986	39	Mercuric chloride 0.1%	5	3	60.0
	39	Chlorox 20%, 20 mins	8	6	75.0
	39	Dipped in 70% ethyl alcohol for 45 secs, then sterilized in 20% chlorox, 10 mins.	10	10	100.0
6-8 October 1986	93	Mercuric chloride 0.1%	10	4	40.0
	93	Chlorox 20%, 20 mins	9	7	77.8
	93	Dipped in 70% ethyl alcohol for 45 secs, then sterilized in 20% chlorox, 10 mins.	9	6	66.7
6-8 October 1986	181	Mercuric chloride 0.1%	10	7	70.0
	181	Chlorox 20%, 20 mins	7	4	57.1
	181	Dipped in 70% ethyl alcohol for 45 secs, then sterilized in 20% chlorox, 10 mins.	8	8	100.0

### 3.2 PART 2 : CALLUS CULTURE

#### 3.2.1 STUDY 2.1 : Callus Culture

##### 3.2.1.1 Objectives :

##### 3.2.1.2 Methods

During this study period, efforts were made to multiply callus from two sources of tissue.

1. From previously maintained callus derived from stems of in vitro - grown seedlings, and
2. From clonal bud cultures.

Despite of the serious problem on contamination during their dormant period, some of them survived (see Table 4) and produced callus at each bud. The callus produced at the cut end of these superior clonal buds were removed from the original cultures and excised into pieces of 5 x 5 mm. and transferred onto agar medium containing the same constituents and the medium for culturing shootlets, but half strenght of macro-nutrients and devoid of auxin whereas BAP was added at 6 mg/l, and kept under continuous light as mentioned earlier.

A total of 17 callus clones were obtained from the survived buds. The quantity of callus in flasks and pieces, and also general observation were shown in Table 4. It should be stated that the callus obtained were, in general, not healthy. This problem must be further solved. However, the callus growth in terms of fresh weight and dry weight had not been recorded since the initial callus were kept for multiplication.

### 3.2.1.3 Future Study for the Following Period

3.2.1.3.1 The clonal callus cultures and also that from seedling explants from previous cultures will be multiplied to provide sufficient materials for future study such as amino acid, inositol and coconut milk experiments as shown in the research proposal 6.2.5 and 6.2.7.

3.2.1.3.2 Some of the callus will be used for histological work.

Table 4 Quantity and quality of two-week old callus prepared for further study

Clone	Quantity		Growth	Colour
	Flask	Callus pieces		
V 25	4	28	+++	yellow, some brown
V 27	3	24	+	blackish brown
V 28	4	29	+++	yellow, blackish brown at base
V 30	4	26	++++	yellow, blackish brown at base
V 35	2	7	+	yellow, blackish brown at base
V 38	2	11	+++	yellow, blackish brown at base
V 40	3	18	+++	brown
V 41	1	7	++	brown
V 43	1	6	++	blackish brown
V 45	3	23	+	brown
V 46	2	8	+	brown
V 49	2	15	+	yellow, black at base
V 69	2	13	+++	yellow, black at base
V 71	9	73	+++	blackish brown
V 72	8	62	+++	yellow, some green
V 78	1	5	+++	brown
V 126	1	4	+++	brown
Callus from stem of sterile seedlings	25	200	+++	brownish yellow

Note :  
 + = poor growth  
 ++ = fair  
 +++ = good  
 ++++ = very good

**3.2.2 STUDY 2.2 : Histological Study**

**3.2.2.1 Objective :** To observe phylotaxy of different clones of teak and histology of callus derived from different cultures.

**3.2.2.2 Materials and Methods :** Shoot tips of different teak clones i.e. V108, V116, V129, V145, V150 and V160 and fleshy as well as dried secondary callus and woody callus derived from the cultures of V101 clone were fixed in a solution of FAA (70% ethanol, glacial acetic acid, formalin at 90:5:5). Dehydration of those fixed specimens was carried out by tertiary butyl alcohol method before embedding in paraffin as described by Gray (1972), Johansen (1940), Peacock (1940), and Steedman (1960).

At present, embedded specimens as described above are in the process of microtome sectioning, mounting and staining, respectively. Histological studies of the sectioned specimens will be carried out as soon as such process is completed.

### 3.3 PART 3 : TEAK ANTHOR CULTURE

#### 3.3.1 OBJECTIVE :

The objective of this study is to establish techniques of anther culture for future application in the teak improvement programme.

#### 3.3.2 MATERIALS :

Plant materials (anthers) used in this series of studies were from both the trees in the CMU campus and clones in the TIC breeding orchard. The flowering period of teak is July-September. At each collection time, the inflorescences were detached from the trees, put in sealed plastic bags and kept in an ice box for transportation. In the laboratory, they were stored in a refrigerator for the studies.

The studies were conducted in the laboratory of Department of Biological Science, Faculty of Science, CMU.

#### 3.3.3 STUDY 3.1 : Flower Buds for Anther Culture

##### 3.3.3.1 Objective :

The objective of this study is to identify the most

suitable size of teak flower buds (unopened buds) containing anthers suitable for the anther culture studies.

### 3.3.3.2 Methods :

Teak flowers (inflorescences) used in this study came from the trees in the CMU campus. The inflorescences were detached from the donor trees and brought to the laboratory. The characteristics and structures of flowers, flower buds and the developmental stages of anthers and pollen, i.e. meiosis, uninucleate and binucleate stages, which are suitable for the anther culture studies were studied under the microscopic conditions.

The staining techniques used for cytological studies are the aceto-orcein, aceto-carmin, propionio-orcein and Feulgen squash techniques which developed by Darlington and La Cour (1970).

### 3.3.3.3 Results :

3.3.3.3.1 Teak flowers throughout the rainy season (July-September). The flowers are scattered in large panicle. One inflorescence contains over a thousand flower buds but not all of these buds develop at the same time. As a result, there are various stages of flower buds, both flowers and fruits

developed in each inflorescence.

3.3.3.3.2 Teak flower is a perfect flower. The flower size is 6-8 mm in diameter and composed of green calyx (6 sepals), white corolla (6 petals), 6 stamens (6 anthers) and a pistil. The pistil is composed of an ovary (4 ovules) and a styl

3.3.3.3.3 The flower buds (the unopened buds) which are most suitable for the anther culture study should be the brown buds of sizes 1.5-2.0 mm in diameter. Under the microscopic condition, the anthers of these buds are yellow jelly containing the uninucleate microspores (Plate 6).



Plate 6 Teak flower buds used for anther culture.

3.3.3.3.4 The smaller buds (<1.5 mm) contain green jelly anthers in which the sporocytes are in the meiosis stage.

3.3.3.3.5 The bigger buds (>2.0 mm), their anthers are generally dense and yellow containing binucleate grains.

3.3.4 STUDY 3.2 : Preliminary Study on the Development of Anther Culture Media

3.3.4.1 Objective :

The objective of this study is to preliminarily examine various types of culture conditions and culture media on the response of teak anther under in vitro cultures.

3.3.4.2 Method :

Teak flower buds of size 1.5-2.0 mm from the CMU campus trees were used in this study. The selected flower buds were shaken in 10 % (v/v) Chlorox solution for 10 minutes for surface sterilization and then rinsed 3 times in sterile distilled water.

The surface sterilized flower buds were dissected under microscopic/aseptic conditions. The anthers were removed from the dissected buds and transferred onto agar of tested culture media in 50 ml-flasked or bottles.

The cultures were kept at 28<sup>o</sup> C with 10-hour illumination of approximately 3,000 lux supplied by the fluorescent lamps.

The tested basal culture media used in this study were those of W (White, 1943), MS (Murashige and Skoog, 1962), H (Bougain and Nitsch, 1967), SH (Schenk and Hildebrandt, 1972) and B5 (Gamborg and Wetter, 1968) media, with 5 % (w/v) agar and (5 % (w/v) sucrose. The pH of the prepared media were adjusted to the range of 5.7-5.9 and autoclaved at 15 lb/sq.in for 15 minutes.

The growth regulators and their combinations tested in each culture medium were shown in Table 5.

There were 5 anthers per culture flask/bottle and 12 flasks/bottles per treatment (culture medium x growth regulator combination).

The responses of the culture anthers were observed for 90 days.

#### 3.3.4.3 Results :

The results of this study are shown in the following table (Table 5).

Table 5 Effects of basal culture media, growth regulators and their combinations on teak anther culture.

Growth Regulator (mg/l)	Callus Production				
	W	MS	H	B5	SH
0.1 K + 0.5 2,4-D	-	+	-	-	-
0.1 K + 1.0 2,4-D	-	-	-	-	-
0.1 K + 2.0 2,4-D	-	-	-	-	-
0.1 K + 0.5 pCPA	-	-	-	-	-
0.1 K + 1.0 pCPA	-	-	-	-	-
0.1 K + 2.0 pCPA	-	+	-	+	+#
0.1 K + 0.5 NAA	-	-	-	-	-
0.1 K + 1.0 NAA	-	-	-	-	-
0.1 K + 2.0 NAA	-	-	-	-	-
0.1 BA + 2.0 pCPA	-	-	-	-	+

- no callus formation

+ callus formed

# some degree of pollen division were observed

The results of this study indicate that :

3.3.4.3.1 On MS basal medium the supplement of auxin (2,4-D) and cytokinin K (kinetin), and B5 and SH basal media + pCPA (2 mg/l) in a cytokinin containing medium, i.e. kinetin or BA, can be used for callus induction in the teak anther cultures, and

3.3.4.3.2 The combination of 0.1 mg/l of K and 2.0 mg/l of pCPA appears to be the most effective growth regulators among the tested ones for pollen growth and callus formation of

the cultured anthers (Plate 7 and Plate 8)

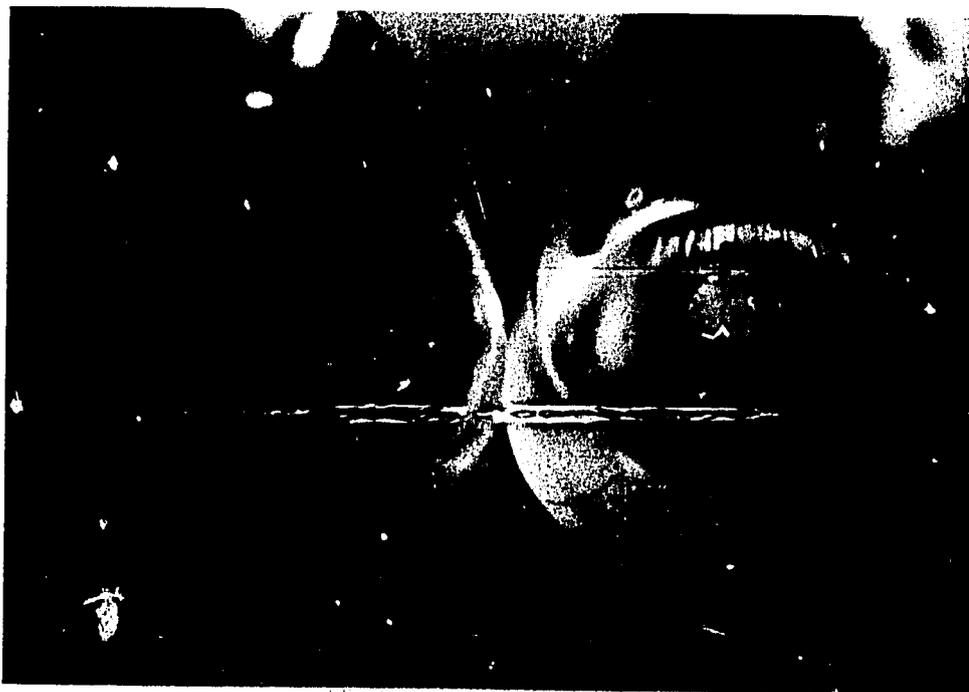


Plate 7 Teak anther culture.



Plate 8 Callus formation and development from anther culture.

**3.3.5 STUDY 3.3 : Cytological Study in Anther Culture**

**3.3.5.1 Objective :**

The objectives of this study are :

3.3.5.1.1 To observe pollen mitoses under the in vitro anther culture, and

3.3.5.1.2 To check chromosome number of the growing callus derived from the anther culture in comparison with that of the sporocytes of the flower buds.

**3.3.5.2 Methods :**

One-week old callus derived from anther cultures in Study 3.2 and the flower buds as described in Study 3.1 were used in this study. Both callus cells and the flower bud anthers were stained for microscopic studies by using the aceto-orcein squash technique as developed by Darlington and La Cour (1970).

### 3.3.5.3 Results :

The results of this study indicate that:

3.3.5.3.1 The division of uninucleate microspores in the teak anthers can occurred under in vitro anther culture (Plate 9) and its chromosome numbers (Plate 10).

3.3.5.3.2 the chromosome number of the growing callus derived from anther culture (Plate 11) is very similar to that of the sporocytes in the flower bud anthers (Plate 10), i.e.  $n = 16$ , and

3.3.5.3.3 the callus derived from the anther culture is also a haploid callus.

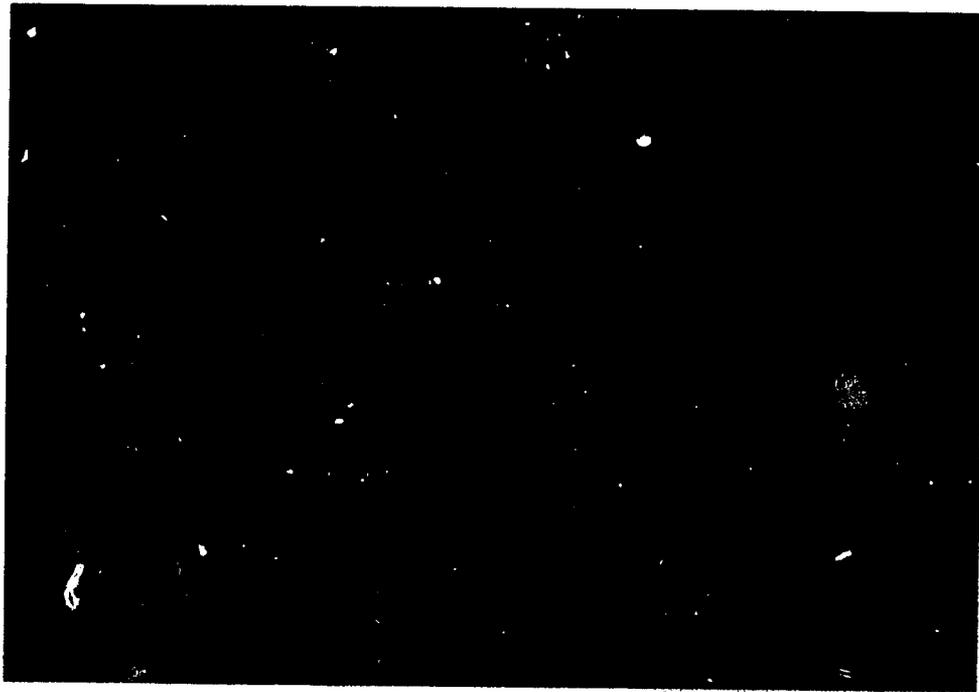


Plate 9 Pollen mitoses in in vitro culture. One pollen (left) was binucleate with one nucleus at metaphase (--->). The pollen on the right was trinucleate with one nucleus at metaphase (--->). Acetic-orcein squash technique ( x 1,000).



Plate 10 Chromosome number of teak developing anther in flower buds (n = 16).

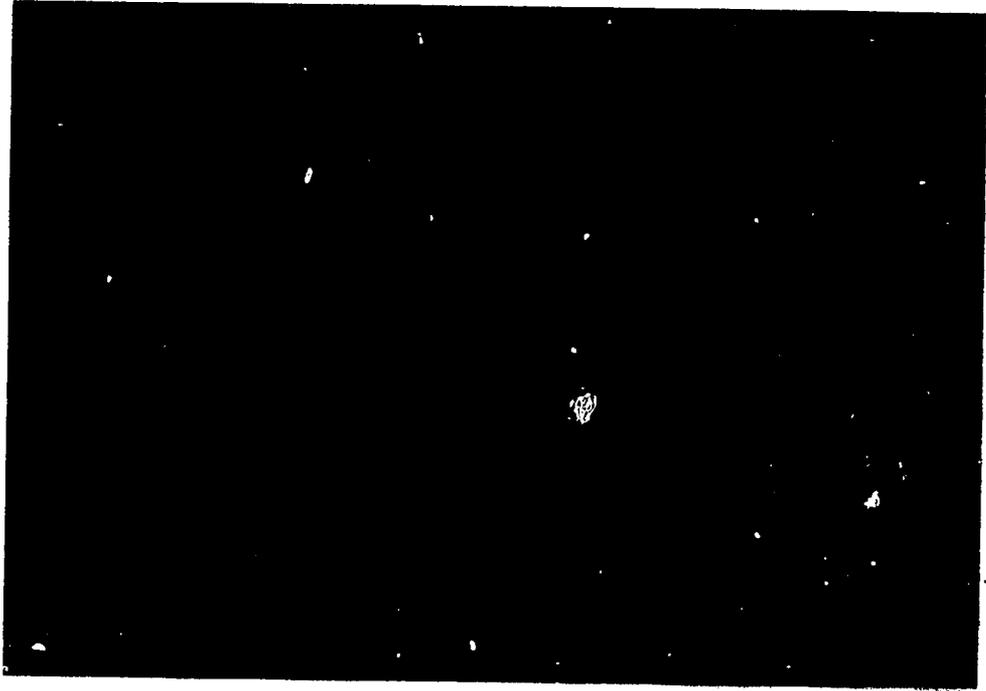


Plate 11      Callus cells of teak where  $n = 16$ .  
(Acetic-orcein squash technique ( x 1,000)).

**3.3.6 STUDY 3.4 : Effects of Adenine Sulfate and the Modified SH Medium**

**3.3.6.1 Objective :**

To study the response of anther to the modified SH medium (which is the most effective medium as indicated in Study 3.2) containing different concentrations of adenine sulfate.

**3.3.6.2 Methods :**

The composition of the medium used in this study was SH macronutrients supplemented with the following additives :

B5 micronutrients	kinetin	0.1 mg/l
B5 vitamins	pCPA	2.0 mg/l
	glycine	2.0 mg/l
	agar	5.0 gm/l
	sucrose	5.0 gm/l

Adenine sulfate was also added at different concentrations, i.e. 0, 1.0, 5.0, 10.0 and 20.0 mg/l, respectively

The responses of the cultured anthers, in term of callus formation, were observed and recorded throughout the period of 1-8 weeks after culturing.

**3.3.6.3 Results :**

The results of this study are summarized as follows:

Table 6 Effects of adenine sulfate on the responses of cultured anther.

Adenine Sulfate (mg/l)	Anther Response (%)	Callus Formation after Culturing (days)	Callus Growth	Callus Condition
0	47	20	++	yellowish, friable
1	27	21	+	yellowish, friable
5	35	24	++	green-yellowish, friable
10	33	24	++	green-yellowish, friable
20	57	24	+++	brown-greenish, friable

Note :  
 + poor callus growth,  
 ++ good callus growth,  
 +++ very good callus growth

The results presented in Table 6 show that :

3.3.6.3.1 Adenine sulfate has no marked effects on the formation of callus of the cultured anthers (in terms of both percentage of cultured anthers that formed callus and time of callus formation after culturing),

3.3.6.3.2 However, this substance showed some effect on growth and the induction of chlorophyll formation of the formed callus.

### 3.3.7 STUDY 3.5 : Preliminary Study on Anther Storage

#### 3.3.7.1 Objective :

The objective of this study is to observe the effect of cold (refrigerator) storage of flower buds on survival, growth and development of the cultured anthers.

#### 3.3.7.2 Method :

Teak flower buds from a CMU campus tree were used in this study. The buds suitable for anther culture were selected, lined on the wet filter paper in petridishes and kept in a refrigerator at temperature of 4-5 C for 3, 5, 7, 14 and 21 days respectively. At each culturing time, i.e. 3, 5, 7, 14 and 21 days after storage, the cold stored flower buds and the fresh flower buds (the control) from the same tree were excised and their anthers were cultured on the SH medium supplemented with 0.1 mg/l of kinetin, 2.0 mg/l of pCPA and 2.0 mg/l of glycine.

The responses of cultured anthers were observed throughout the period of 8 weeks after culturing.

#### 3.3.7.3 Results :

Under the storage conditions tested in this study, it is shown in Table 7 that :

3.3.7.3.1 the flower buds can be stored without deterioration for a period of 3 days,

3.3.7.3.2 the viability of anthers or the response of cultured anthers decreases markedly with an increase in storage period, i.e. from day 0 to day 7, and

3.3.7.3.3 the anther viability will be very poor if the flower buds are stored longer than 7 days.

3.3.7.3.4 the callus condition is very good if the flower buds has been kept in cold storage from 0-7 days whereas longer storage time resulted in poor callus growth.

Table 7 Effects of cold storage on survival, growth and development of the cultured anthers.

Storage Period (days)	Anther Response (%)	Callus Formation after culturing (days)	Callus Condition
0	65	14	+++
3	67	15	+++
5	52	14	+++
7	35	16	+++
14	3	21	+
21	0	-	-

3.3.8 Future Study for the Next Period :

3.3.8.1 Improvement of the developed teak anther culture media for organogenesis in the anther-derived callus culture.

3.3.8.2 Effects of amino acids and dark treatments on the responses of teak cultured anthers.

3.3.8.3 Effects of sucrose concentrations in combination with incubation treatments on the responses of teak cultured anthers.

3.3.8.4 Effects of pCPA concentrations in combination with incubation treatments on the responses of teak cultured anthers.

3.3.8.5 Effects of incubation period on the responses of teak cultured anthers.

3.3.8.6 Effects of clones and flower bud storage on the responses cultured anthers.



in width, 16 m in length and 2.60 m in height. Both houses were designed for the planted materials exposed to more light intensity than those previously received (Plate 12).

An effort was put to fix mist spray systems in both houses to keep favourable humidity and temperature.



Plate 12 Glass-house being constructed for future rooting of shootlets.

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