



UNIVERSITI PUTRA MALAYSIA

**IN VITRO PROPAGATION STUDIES ON THE INITIAL AND
ESTABLISHMENT STAGES OF RUBBER (HEVEA BRASILIENSIS
MUELL. ARG.)**

ALEJANDRO FLORES RODRIGUEZ

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ESTABLISHMENT STAGES OF RUBBER (*HEVEA BRASILIENSIS*
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By

ALEJANDRO FLORES RODRIGUEZ

**Thesis Submitted in Fulfilment of the Requirements for the Degree of Master of
Agricultural Science in the Faculty of Agriculture,
Universiti Pertanian Malaysia.**

February 1997



DEDICATION

I find great delight in being able to dedicate this thesis, firstly, to my Lord Jesus Christ. Secondly, the dedication goes to the persons that give meaning to my existence:

My dear mother Mrs. Reyna Rodriguez for all her sacrifices.

My beloved spouse Mrs. Carmen Sainz for being so understanding and encouraging.

My very much adored daughter Anna Karen, the little person who *de facto* motivated all this odyssey and to date keeps on lightening up my life.

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LIST OF ABBREVIATIONS

AC	: activated charcoal
AOX	: antioxidant solution (15 mg/L ascorbic acid + 10 mg/L citric acid)
BA (BAP)	: N ⁶ -benzylaminopurine
DDW	: sterile double distilled water
DF	: degrees of freedom
DMSO	: dimethylsulfoxide
EDTA	: ethylenediaminetetraacetic acid
GA ₃	: gibberellic acid
GT 1	: Gondang Tapen rubber clone No. 1
HgCl ₂	: mercuric chloride
IBA	: indole-3-butyric acid
MS-	: Murashige & Skoog's basic formulation medium (half strength)
MS+AC	: MS- medium modified with activated charcoal
MS+AOX	: MS- medium & preculture soaking in antioxidant solution
MS+SN4	: MS- medium modified with silver nitrate (4 mg/L)
MS+SN6	: MS- medium modified with silver nitrate (6 mg/L)
NAA	: α-naphtaleneacetic acid
PPZ	: 3-methyl-1-phenyl-5-pyrazolone
RRIM	: Rubber Research Institute of Malaysia
RRIM 600	: Rubber Research Institute of Malaysia rubber clone No. 600
RRIM 623	: Rubber Research Institute of Malaysia rubber clone No. 623
SN	: silver nitrate
2,4-D	: 2,4-diclorophenoxyacetic acid



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FEBRUARY 1997

Chairman : Associate Prof. Dr. Saleh Kadzimin

Faculty : Agriculture

Development of *in vitro* methods to propagate rubber is highly desirable. Some procedures exist but they generally do not address well the initial and establishment stages. This leads to minimal arrest of two common deterrents in any micropropagation system, i.e., contamination and browning. This project attempted to study both contamination and browning phenomena with an aim of improving survival without altering regeneration of explants.

Field materials of clone RRIM 600 were used. Experiments on the Initial and Establishment stages, their interactions and concluding experiments were conducted. In all experiments, explants were cultured in the standard tissue culture procedures on basic MS medium.



In the Initial stage, the results showed no significant difference between chemical treatments of antibiotics and/or fungicide in the field and control. As for the physical characteristics, efficient initial measures were to use 6-week old middle portions or shoot tips. This could be improved by growing these explants in a glasshouse.

In the Establishment stage, the most efficient procedure to decontaminate explants was Procedure E that included a succession of treatments with ethanol, Clorox, Rifampicin, Ampicillin, DMSO and Triton-X before culturing. Regarding debrowning treatments, the best overall responses were obtained by culturing explants in MS medium (half strength) modified by the addition of 4 mg/L silver nitrate.

In the analysis of the interactions between treatments in the Initial and Establishment stages, results showed that the combination of treatments with better potential was the one that included the use of mature explants (6-week old) surface-decontaminated by Procedure E.

The concluding experiments included significant treatments taken from the results of preceding experiments. The most effective protocol included the use of 6-week old shoot tips as explants surface-decontaminated by Procedure E. At this point, the effectiveness of the treatment was enhanced by soaking explants in a



solution made up of Benlate and Rifampicin and cultured in MS medium (half strength) modified with 4 mg/L silver nitrate.

Growing source plants of clone RRIM 600 in the glasshouse and using the latter concluding protocol allowed for 100% axenic viable cultures with 84% regeneration especially for mature explants.



Abstrak tesis ini diserahkan kepada Senat Universiti Pertanian Malaysia sebagaimemenuhi keperluan untuk penganugerahan ijazah Master Sains Pertanian.

**KAJIAN PEMBIAKAN *IN VITRO* MENGENAI PERINGKAT-PERINGKAT
PERMULAAN DAN PEMBENTUKAN DALAM TANAMAN
GETAH (*HEVEA BRASILIENSIS* MUELL. ARG.)**

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Perkembangan kaedah *in vitro* dalam pembiakan getah adalah sangat diperlukan. Kaedah-kaedah yang sedia ada secara amnya tidak dapat menerangkan dengan jelas peringkat-peringkat permulaan dan pembentukan. Ini membawa kepada kurangnya pemahaman mengenai masalah yang biasa wujud dalam sistem pembiakan mikro iaitu kontaminasi dan pemerangan. Projek ini adalah untuk mengkaji fenomena kontaminasi dan pemerangan dengan tujuan untuk memperbaiki keupayaan eksplan untuk hidup tanpa mengubah penjanaan semula.

Bahan tanaman dari ladang yang terdiri daripada klon RRIM 600 telah digunakan. Ujikaji pada peringkat permulaan dan pembentukan, interaksi di antara kedua-duanya dan ujikaji menyeluruh telah dijalankan. Dalam semua ujikaji, eksplan



kedua-duanya dan ujikaji menyeluruh telah dijalankan. Dalam semua ujikaji, eksplan telah dikulturkan mengikut kaedah-kaedah kultur tisu yang standard di atas medium MS asas.

Dalam peringkat permulaan, keputusan menunjukkan tiada perbezaan yang bererti di antara rawatan kimia dengan antibiotik dan/atau racun kulat di ladang berbanding kawalan. Untuk penilaian ciri-ciri fizikal dalam kajian ini, ukuran permulaan yang berkesan adalah dengan menggunakan mercu pucuk atau bahagian tengah yang berusia 6 minggu. Ini boleh diperbaiki dengan menanam sumber eksplan di rumah kaca.

Dalam peringkat pembentukan, kaedah yang paling berkesan untuk mencegah kontaminasi eksplan adalah Kaedah E yang merangkumi berturutan rawatan ethanol, Clorox, Rifampicin, Ampicillin, DMSO dan Triton-X sebelum dikulturkan. Manakala reaksi keseluruhan yang terbaik bagi rawatan untuk mencegah pemerangan diperolehi dengan mengkultur eksplan dalam medium MS (separuh kekuatan) yang diubahsuai dengan penambahan 4 mg/L argentum nitrat.

Dalam analisis interaksi di antara rawatan di dalam peringkat permulaan dan pembentukan, ia menunjukkan bahawa kombinasi rawatan-rawatan yang mempunyai potensi yang lebih baik adalah kombinasi yang menggunakan eksplan yang matang (6 minggu) serta Kaedah E sebagai pencegah kontaminasi permukaan.

Ujikaji menyeluruh termasuklah rawatan-rawatan yang bererti yang di ambil dari keputusan ujikaji-ujikaji terdahulu. Protokol yang paling berkesan adalah penggunaan eksplan dari mercu pucuk yang berusia 6 minggu serta Kaedah E sebagai pencegah kontaminasi permukaan. Pada tahap ini, keberkesanan rawatan didapati meningkat dengan merendam eksplan dalam larutan yang terdiri daripada Benlate dan Rifampicin dan dikulturkan dalam medium MS (separuh kekuatan) yang diubahsuai dengan 4 mg/L argentum nitrat.

Menanam pokok induk dari klon RRIM 600 di rumah kaca dan menggunakan protokol menyeluruh membolehkan pencapaian 100% kultur aksenik dengan 84% penjanaan semula terutama sekali bagi eksplan yang matang.

CHAPTER I

INTRODUCTION

Natural rubber was first identified by the early European explorers of the American continent as the ingredient of the ball used by the Maya and Olmec people in their sacred game. Today, despite competition with synthetic polymers, natural rubber remains indispensable for the manufacture of many products mainly due to its physical properties.

Natural rubber exists in many plants and trees as latex but the main source is from the rubber tree *Hevea brasiliensis* Muell. Arg. This species is indigenous to the Amazon basin of South America and is under culture in almost all tropical and some subtropical areas in the world.

Propagation of rubber for commercial planting was done mainly by seeds, but due to its heterozygous nature, its genuineness could not be maintained. Today, rubber is commercially propagated by grafting buds from selected genotypes onto rootstocks of mixed origin. Some variations remain and they are mainly attributed to



the rootstock effect. This hampers the full expression of new genotypes potential generating a gap for improvement via vegetative propagation.

The fact justifies every effort to obtain true vegetative propagation avoiding the disadvantages of the partial propagation (grafting). *In vitro* culture appears to be an ideal solution for rubber plantations of high yielding trees.

The priority in *in vitro* propagation system for *Hevea* is to produce a new type of plant material that makes better use of the improvements made by the breeders. The *in vitro* propagation system through the use of axillary shoots appears to be the most suitable method as it maintains genetic stability far better than any other methods.

However, tissue culture demands rigorous exclusion of contaminating microorganisms. All nutrient media, culture vessels, instruments used in handling tissues and the plant material itself, must be contaminant-free. This is particularly important when working with woody plant species such as rubber. Endogenous microbial contamination of plants and their stem tissues preclude the establishment of rubber as a tissue culture system. High rates of contamination (60-100%) occur when mother trees are grown in tropical or subtropical regions, which is the normal and most realistic situation for this species. This is particularly true when working with clonal materials.

In addition, excision that causes wounding initiates the production of polyphenol oxidases that cause browning of the tissue affecting its metabolism in various ways leading to death. In rubber, this situation appears to be one of the greatest problems in the culture of its tissues.

A search in the literature reveals that there are very limited studies concerning the initial stages of *in-vitro* propagation of this crop. The present study is set to carry out basic investigations on the initial stage of *in vitro* propagation as well as the establishment of viable axenic cultures.

The main objective of the study is to examine the effects of different procedures of decontamination and debrowning at different stages of culture of clonal explants. The main focus is on contamination and browning as well as survival and regeneration parameters. The present study attempts to improve the efficiency of decontamination and debrowning procedures during the initial stages of micropropagation of rubber establishing a more reliable baseline for further studies.

CHAPTER II

LITERATURE REVIEW

Stages of *In Vitro* Plant Propagation Systems

It is well known that plant tissue culture consists of three stages as proposed by Murashige (1974a, b, 1976, 1978). These stages include, establishment of the axenic culture, multiplication of propagula for *in vitro* plant propagation and preparation for transfer of plant to soil.

Debergh and Read (1991) however proposed a different scheme focusing in the pre-culture phase to obtain healthier explants and uniform responses. Their main justification is that for many plant species it is of most importance to avoid contamination than it is to induce the first reaction. They added that rooting procedure should also change making it more economical and practical. With these considerations, their procedures consist of the following stages:

Stage 0: The preparation of stock plants under hygienic conditions;

Stage 1: The establishment of aseptic cultures;



Stage 2: The induction of meristematic centres, their development into buds and their rapid multiplication;

Stage 3: The elongation of the buds to shoots and the preparation of uniform shoots for stage 4;

Stage 4: The rooting and the initial growth of the *in vitro* produced shoots under *in vivo* conditions.

Stage 0: The Initial Stage

Many authors support the scheme proposed by Debergh and Read (1991) and conclude that the success or failure of micropropagation of tree species often depends on the condition of the plant material at the time of collection. Environmental factors also influence the level of microbial contamination of plant tissues (Bonga and von Aderkas, 1992). Because of the unpredictable effects of the external environment on the physiological condition of plants, many researchers prefer to use material grown in glasshouses. In a glasshouse situation, one can optimise plant vigour reasonably high by using optimal light, temperature and fertilization schedules (Read, 1987; Anderson, 1980). Furthermore, strict pest control is easier in the glasshouse than in the open field. Because of this, explants from glasshouse-grown plants are often less contaminated than those from field-grown plants (McCullouch and Briggs, 1982). For tropical and subtropical ornamentals, it is advisable to maintain a relatively high temperature (25⁰ C) and a relatively low humidity (75%); for nursery plants, a plastic glasshouse is preferred but it is not necessary to control temperature and relative

humidity (Debergh and Read, 1991). These authors also stated that the impact of Stage 0 is apparently not limited to the sanitary situation of the explants in Stage 1, but it also influences the rate of survival. For example, before effecting Stage 0, success with *Cordyline spp.*, was limited to the development of apical buds only. All cross sections of the stem bearing nodal tissue were either contaminated, or did not react consistently to produce propagules for Stage 2 (Miller and Murashige, 1976). It was found that, to avoid contamination, the stem had to be defoliated and cleaned prior to surface sterilisation. After Stage 0 was effected, it was possible to use a sterilisation procedure for stem tissue with leaf bases still attached, without having to completely defoliate the stems. Thus each nodal section of the stem as well as the terminal shoot tips could be used as primary explant. The results were completely different. All the explants produced shoots usable for subculture (Debergh and Read, 1991).

Contaminants found in micropropagation systems are introduced either through carry-over on the surface or in the tissue of explants, or through deficient procedures in the laboratory. It has been known that plant surfaces are habitats for microorganisms (Campbell, 1985). During plant growth and development, many microorganisms enter the tissues of plants through natural openings or wounds, later colonising the plant tissues. Facultative and obligate pathogens colonise plants by vector-assisted or host-plant penetration mechanisms (Tarr, 1972; Matthews, 1981). The implication is that plants develop an endophytic 'assortment' of variable species composition consisting of inter-and intracellular microorganisms including viruses,

viroids, prokaryotes (bacterial and bacteria-like agents) and fungi. In establishing tissue cultures using leaf, petiole or stem explant, most, if not all, surface and endophytic microorganisms may be carried over (Cassells, 1991).

Given the range of microorganisms colonising plant tissues and the diversity of plant pathogens, it is not extraordinary that research has shown the appearance of intercellular microorganisms in every tissue of plants (Bastiaens, 1983). Bastiaens (1983) also confirmed that this is especially true of plants in long-term propagation or in areas of high biological diversity. Plants grown annually from seeds tend to be less heavily contaminated. Likewise, long term species have a tendency to accumulate intracellular microorganisms.

In conclusion, Stage 0 is an effective remedy for contamination problems and it makes explants more reliable as starting material.

Stage 1: The Establishment of Aseptic Cultures

The purpose of this stage is to initiate axenic cultures. Success at this stage firstly requires that explants should be safely transferred to the culture environment and secondly, that there should be an appropriate reaction (e.g., growth of a shoot tip or formation of callus on a stem piece). Usually a batch of explants is transferred to culture at the same time. Stage 1 would be regarded as satisfactorily completed if an

adequate number of them survived without contamination and was growing (George and Sherrington, 1984).

Generally, surface sterilisation of juvenile material is not difficult compared to older trees. Explants from branches of 5-year-old *Eucalyptus grandis* were all found to be contaminated after surface sterilisation while more than half of those taken from its juvenile sprouts were clean (Warrag et al. 1990).

For most micropropagation work, the explant of choice is an apical or axillary bud. For only a limited number of plants other explants are used, e.g., leaf apices of *Ficus lyrata* (Debergh and De Wael, 1977), *Anthurium spp.*, (Pierik and Steegmans, 1976), *Saintpaulia ionantha* (Margara and Piollat, 1985), *Gloxinia* (Johnson, 1978) and flower heads of *Gerbera jamesonii* (Pierik et al., 1975). It is always more risky to start a micropropagation scheme with the *de novo* formation of buds as the chances of obtaining off-type plants are higher (Debergh and Maene, 1989).

The developmental stage of an explant can be of tremendous importance. The age of the stock plant, the physiological age of the explant and its developmental stage, as well as its size can determine the success of a procedure. More often the only way to obtain contamination-free cultures is by culturing apical shoot meristems which are the smallest explants available. For example, apical meristems of mature *Juglans nigra x regia* trees were free of contaminants whereas stem sections were severely contaminated (Meynierd and Arnould, 1989).