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IN VITRO CULTURE ESTABLISHMENT AND SHOOT REGENERATION IN RUBBER (HEVEA BRASILIENSIS MUELL. ARG.)

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By

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Chairman: Associate Professor Maheran Abdul Aziz, PhD
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The development of *in vitro* methods to produce high quality clonal planting materials of *Hevea brasiliensis* for replanting and new planting is highly desirable and essential. Some procedures do exist but generally do not address well the initial stage of culture establishment. The establishment of *in vitro* cultures of challenging woody plant species like *H. brasiliensis* have frequently been hampered by persistent microbial contamination, phenolic production, phase change and low response to media. Therefore, the overall goal of this study was to establish an efficient *in vitro* propagation method for *H. brasiliensis* through solving the persistent microbial contamination problems, controlling phenolic production and increasing the response of explants to media. Shoot tips and axillary buds derived from one to two year-old grafted plants of *H. brasiliensis* clone RRIM 2025 were used as explants. In determining the most suitable method of reducing explant contamination, different concentrations of sodium hypochlorite, mercuric chloride and nano silver at different immersion times were assessed on explants derived from three physiological leaf stages (bronze, light green and stable green leaf stages) of preculture and non-preculture treated plants. The highest percentage of survival (94.44%) was from explants derived from the light green leaf stage of preculture treated plants immersed in 92.6 µM NS for 20 min. Different types of adsorbents which were silver nitrate, activated charcoal and nano silver were assessed in controlling browning of *in vitro* cultures of *H. brasiliensis.* Nano silver at 37.04 µM significantly produced the highest percentage of explant survival (87.03%) with a low percentage of browning (10%). In an attempt to determine the most suitable medium for *in vitro* culture of *H. brasiliensis* explants, Murashige and Skoog (MS) medium, Woody Plant medium (WPM) and MB medium in combination with 3%, 6% and 9% sucrose concentrations were assessed. After 16 weeks of culture, the highest percentage of shoot formation (80%) was on MB medium with 6% sucrose. In evaluating the effects of benzyl aminopurine (BAP) alone on shoot induction of *H. brasiliensis,* the highest percentage of

axillary shoot emergence (61.11%) was obtained on MB medium containing 22.2 µM BAP after 16 weeks of culture. In the second experiment on shoot induction, different concentrations of BAP in combination with 1.44 µM Gibberellic acid (GA_3) were assessed whereby MB medium containing 22.2 µM BAP with 1.44 µM GA_3 produced a maximum mean number of 2 shoots per explant after 16 weeks of culture. Lastly, different concentrations of BAP in combination with 2.7 µM naphthalene acetic acid (NAA) were assessed on shoot induction whereby the highest mean number of 3 shoots per explant were obtained on MB medium supplemented 8.8 µM BAP and 2.7 µM NAA after 16 weeks of culture. In the study on multiplication and elongation of *H. brasiliensis* shoots, various combinations of plant growth regulators were assessed. In the first experiment, different concentrations of GA_3 in combination with 2.2 µM BAP and 1.23 µM indole-3-butyric acid (IBA) were tested and a maximum mean number of 10 shoots was produced on MB medium supplemented with 1.45 µM GA_3, 2.2 µM BAP and 1.23 µM IBA after 16 weeks of culture. In the second shoot multiplication experiment, *in vitro* shoots were placed in different concentrations of thidiazuron (TDZ) combined with 0.1 µM IBA and the highest mean number of shoots per explant (4.6) was obtained on MB medium containing 0.45 µM TDZ and 0.1 µM IBA after 16 weeks of culture, Finally, different concentrations of kinetin (Kin) in combination with 4.4 µM BAP and 2.7 µM NAA were tested for shoot multiplication and a maximum mean number of 4.33 shoots, which were strong and healthy, was obtained on MB medium supplemented with 9.3 µM Kin, 4.4 µM BAP and 2.7 µM NAA. In conclusion, this study indicates that a successful establishment of *in vitro* culture of *H. brasiliensis* requires the understanding of the species leaf developmental stages, the interaction of the explant source with the environment and the effective application of the non-toxic silver nano particles in reducing microbial contamination and browning of *Hevea* explants. An efficient initial culture establishment developed in this study thus opens the way for future application of the microcutting technique for propagation as well as genetic improvement of *H. brasiliensis*.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

PEMBENTUKAN KULTUR *IN VITRO* DAN REGENERASI PUCUK UNTUK TANAMAN GETAH (*HEVEA BRASILIENSIS* MUELL. ARG.)

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Pembangunan kaedah *in vitro* untuk menghasilkan bahan tanaman klon *Hevea brasiliensis* berkualiti tinggi untuk penanaman semula dan penanaman baru adalah sangat wajar dan penting. Beberapa prosedur memang wujud tetapi umumnya tidak menangani dengan baik peringkat awal pembentukan kultur. Pembentukan kultur *in vitro* bagi spesies tumbuhan berkayu yang mencabar seperti *H. brasiliensis* telah kerap dihalang oleh pencemaran mikrob yang berterusan, pengeluaran fenolik, perubahan fasa dan tindak balas yang rendah terhadap media. Oleh itu, matlamat keseluruhan kajian ini adalah untuk membentuk kaedah pembiakan *in vitro* yang berkesan untuk *H. brasiliensis* melalui penyelesaian masalah pencemaran mikrob yang berterusan, mengawal pengeluaran fenolik, dan meningkatkan tindak balas eksplan terhadap media. Hujung pucuk dan tunas aksil yang diperoleh dari pokok cantuman *H. brasiliensis* klon RRIM 2025 berumur satu ke dua tahun, digunakan sebagai eksplan. Dalam menentukan kaedah yang paling sesuai bagi mengurangkan pencemaran eksplan, kepekatan natrium hipoklorit, merkurik klorida dan nano silver (NS) serta masa rendaman yang berbeza telah diuji ke atas eksplan yang diperoleh daripada tiga peringkat fisiologi daun (peringkat daun gangsa, hijau muda dan hijau stabil) daripada pokok yang diberi rawatan prakultur dan tanpa rawatan. Peratusan hidup tertinggi (94.44%) adalah bagi eksplan yang diperoleh daripada peringkat daun hijau muda pokok yang diberi rawatan prakultur setelah direndam dalam 92.6 µM NS selama 20 min. Pelbagai jenis adsorben iaitu silver nitrat, arang teraktif dan NS telah di uji untuk mengawal pemerangan kultur *in vitro H. brasiliensis*. Didapati NS pada 37.04 µM secara signifikan menghasilkan peratusan hidup eksplan tertinggi (87.03%) dengan peratusan pemerangan yang rendah (10%). Dalam usaha menentukan medium yang paling sesuai untuk kultur *in vitro H. brasiliensis*, medium Murashige and Skoog (MS), medium Tumbuhan Berkayu (WPM) dan

medium MB yang digabungkan dengan kepekatan 3%, 6% dan 9% sukrosa telah diuji. Selepas 16 minggu dikultur, peratusan tertinggi pembentukan pucuk (80%) adalah pada medium MB dengan 6% sukrosa. Untuk mengkaji kesan kepekatan benzyl aminopurine (BAP) secara bersendirian ke atas induksi pucuk *H. brasiliensis,* peratusan pengeluaran pucuk aksil tertinggi (61.11%) adalah pada medium MB yang mengandungi 22.2 µM BAP selepas 16 minggu dikultur. Bagi eksperimen induksi pucuk yang kedua, kepekatan BAP yang berbeza beserta 1.44 µM asid Gibberellic (GA_3) telah diuji dan didapai medium MB mengandungi 22.2 µM BAP dengan 1.44 µM GA_3 menghasilkan min tertinggi 2 pucuk per eksplan selepas 16 minggu dikultur. Seterusnya, kepekatan BAP yang berbeza beserta 2.7 µM asid naftalen asetik (NAA) telah diuji untuk induksi pucuk dimana min tertinggi 3 pucuk per eksplan diperoleh di atas medium MB yang mengandungi 8.8 µM BAP dan 2.7 µM NAA selepas 16 minggu dikultur. Dalam kajian penggandaan pucuk *H. brasiliensis,* pelbagai kombinasi pengawalatur tumbesaran tumbuhan telah diuji. Dalam eksperimen pertama, kepekatan GA_3 yang berbeza beserta 2.2 µM BAP dan 1.23 µM asid indol-3-butirik (IBA) telah diuji dan min tertinggi 10 pucuk per eksplan diperoleh pada medium MB mengandungi 1.45 µM GA_3, 2.2 µM BAP dan 1.23 µM IBA selepas 16 minggu dikultur. Bagi eksperimen penggandaan pucuk yang kedua, pucuk *in vitro* telah dikultur pada kepekatan thidiazuron (TDZ) yang berbeza yang digabungkan dengan 0.1 µM IBA dan min bilangan puck tertinggi per eksplan (4.6) diperoleh pada medium MB yang mengandungi 0.45 µM TDZ dan 0.1 µM IBA selepas 16 minggu kultur. Akhir sekali, kepekatan kinetin (Kin) yang berbeza beserta 4.4 µM BAP dan 2.7 µM NAA telah diuji untuk penggandaan pucuk. Rumusannya, kajian ini menunjukkan bahawa kejayaan membangunkan kultur *in vitro H. brasiliensis* memerlukan kefahaman mengenai peringkat pembentukan daun spesies tersebut, interaksi sumber eksplan dengan persekitaran dan aplikasi berkesan partikel nano silver yang tidak toksik bagi mengurangkan pencemaran microbial dan pemerangan eksplan *Hevea.* Pembentukan pengkulturan yang efisien di dalam kajian ini membuka jalan untuk aplikasi teknik keratan mikro bagi pembiakan dan pembaikan genetik *H. brasiliensis* di masa hadapan.

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I certify that a Thesis Examination Committee has met on 28th of May 2015 to conduct the final examination of Mahdi Moradpour on his thesis entitled ***In Vitro* Culture Establishment and Shoot Regeneration in Rubber (*Hevea Brasiliensis* Muell. Arg.)** in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of requirement for degree of Master of Science. The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

µg	Microgram
µM	Micromolar
AC	Activated charcoal
ANOVA	Analysis of variance
BAP	6-Benzyl aminopurine
CaOCl	Sodium hypochlorite
cm	Centimeter
CRD	Complete Randomized Design
DMRT	Duncan multiple range test
GA_3	Gibberellic acid
$HgCl_2$	Mercuric chloride
IBA	Indole-3-butyric acid
Kin	Kinetin
MB	Enjarlic and Carron medium
mg	Milligram
mL	Milliliter
mm	Millimeter
MS	Murashige and Skoog
NAA	α-Naphthalene acetic acid
NS	Nano silver
PGR	Plant Growth Regulator
RCBD	Randomized Complete Block Design
SN	Silver nitrate
TDZ	Thidiazuron
WPM	Woody plant medium

CHAPTER 1

INTRODUCTION

Natural rubber, produced from the latex of the rubber tree *Hevea brasiliensis,* accounts for about 40% of the world's total rubber utilization while 60% is produced by synthetic processes (Lieberei, 2007). The annual world demand for natural rubber is constantly growing because of its distinguishing physicochemical properties, which are still not achievable in synthetic products. World natural rubber production has been steadily increasing since the turn of the century and it currently stands at 11,603,000 tonnes per year (Department of Statistics Malaysia, 2014). Most rubber plantations are located in South East Asia, especially in Thailand, Indonesia and Malaysia, with growing production areas in Vietnam and China (Lieberei, 2007).The increasing demand for natural rubber should persist in the coming decades due to the foreseeable increases in utilization in many countries. The possibilities of extending the planted areas seem limited nowadays and it is primarily through yield increases that growers will be able to satisfy the demand. The quality of the planting material used is an essential component of yield (Jain *et al.,* 2000).

True-to-type multiplication of selected planting materials through cutting has long been troubled with difficulty in developing a cutting technique, due to early loss of rhizogenesis capacity during young tree growth, and the observation of deficient root systems on cuttings (Jain *et al.,* 2000). Rubber trees have been traditionally propagated by grafting buds from selected clones on seedlings or plants from seed orchards. This process is lengthy, since one to two years are required before the plants can be transplanted to the field. Furthermore, no evaluation on the interaction between rootstock and scion has been carried out. For this reason the grafted plants sometimes do not produce the natural rubber at the expected levels (Mendanha *et al*., 1998).

Certain non-uniformity and heterogeneity is seen in bud-grafted clones of rubber, which is particularly ascribable to the non-selected stocks. Such handicaps would be potentially removed by the *in vitro* propagation of plants on their own roots (Carron *et al*., 1989) as well as reduce the cost and time required for plant production. Micropropagation, or propagation *in vitro* of complete plants, is a promising technique for large scale multiplication of selected clones of rubber. The success or failure of micropropagation of tree species including rubber often depends on the condition of the plant material at the time of collection. This is particularly true when explants are obtained from trees grown in the field. The physiological conditions of tissues vary with

season, position within the tree and climatic factors. Each of these conditions can affect the manner in which tissue responds in culture. In addition, environmental conditions have an effect on the degree of microbial infection on plant tissues and hence the degree of contamination would depend on the time explants are collected (Bonga, 1982). Internal and external contamination of plant tissues turns out to be a prevailing problem, because microorganisms, mostly fungi and bacteria, can grow much faster than plant cells and take up all the nutrients, preventing the plants from growing (Cassells, 1990). Although there are techniques to minimize the possibility of bacterial and fungal contaminations during *in vitro* propagation, such as meristem culture and repetitive subcultures, designing a more efficient approach to sterilize plant tissues still seems necessary to eliminate labour intensive trial and errors, and time-consuming decontamination procedures. In order to eliminate the persistent fungal and bacterial contaminations, treatment with antibiotics and antifungal agents may be used. However, it has been reported that antibiotics are normally phytotoxic, and have an inhibitory effect on multiplication, callus induction, regeneration, and explant survival (Teixeira *et al*., 2003).

To date, the *in vitro* culture establishment of *H. brasiliensis* is still problematic. Beside the occurrence of contamination, rapid browning and/or necrosis of the explants is another obstacle. These problems are at least partly caused by oxidation of polyphenols which are abundant in *Hevea* species. Also, inhibition of shoot organogenesis and necrosis of the explants are associated with considerable leakage of exudates into the culture medium (Mederos and Trujillo, 1999). Lack of an optimal protocol for sterilization and debrowning of field-, orchard- or greenhouse-derived (*ex vitro*) explants may result in a paucity of samples for further research (Mahna *et al*., 2013). To optimise an efficient tissue culture method for *Hevea* rubber, explant preparation is one of the important steps that need to be given attention in order to overcome the problem of microbial contamination and browning. A detailed knowledge of mother plant and microbial contamination interaction or host-pathogen combination requires understanding of the dynamics of *Hevea* leaf development and the biochemical potential of cyanide liberation from the species. This study therefore seeks to provide an improved and efficient tissue culture technique for the production of *H. brasiliensis* microcuttings.

The specific objectives of this study were:

1- To determine the effects of sterilant type, preculture treatment and leaf development stage on overcoming contamination of *H. brasiliensis* explants;
2- To determine the effects of different types of adsorbent in controlling browning of *in vitro* culture of *H. brasiliensis;*
3- To optimize the basal medium and plant growth regulator requirements for shoot induction of *H. brasiliensis.*

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LIST Of PUBLICATIONS

Moradpour, M., Aziz, M. A. and Abdullah, S. A. (2014). The Effects of Basic Medium and Sucrose Concentration on Growth of *Hevea Brasiliensis* Microcuttings. Poster presented at UPM- Shizuoka University International Colloquium, Universiti Putra Malaysia, Selangor, Malaysia.

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