Propagation of Rubber (*Hevea brasiliensis* Muell. Arg.) through Somatic Embryogenesis and Assessment of Somaclonal Variation by Molecular Markers

Yupaporn Sirisom

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Plant Science

Prince of Songkla University

2013

Thesis Title Propagation of Rubber (*Hevea brasiliensis* Muell. Arg.) through Somatic Embryogenesis and Assessment of Somaclonal Variation by Molecular Markers

Author Miss Yupaporn Sirisom

Major Program Plant Science

Major Advisor

……

(Assoc.Prof.Dr.Sompong Te-chato)

Examining Committee:

……Chairperson

(Dr.Supawadee Ramasoot)

……

(Assoc.Prof.Dr.Sompong Te-chato)

……

(Assoc.Prof.Dr.Sayan Sdoodee)

The Graduate School, Prince of Songkla University, has approved this thesis as partial fulfillment of the requirements for the Degree of Doctor of Philosophy in Plant Science.

……..

(Assoc.Prof.Dr.Teerapol Srichana)

Dean of Graduate School

This is to certify that the work here submitted is the result of the candidate's own investigations. Due acknowledgement has been made of any assistance received.

_____Signature

(Assoc.Prof.Dr.Sompong Te-chato)

Major Advisor

_____Signature

(Miss Yupaporn Sirisom)

Candidate

I hereby certify that this work has not been accepted in substance for any degree, and is not being currently submitted in candidature for any degree.

_____Signature

(Miss Yupaporn Sirisom)

Candidate

ชื่อวิทยานิพนธ์ การขยายพันธุ์ยางพาราโดยผ่านกระบวนการ Somatic Embryogenesis และตรวจสอบความแปรปรวนทางพันธุกรรมด้วยเครื่องหมายโมเลกุล
ผู้เขียน นางสาวยุพาภรณ์ ศิริโสม
สาขาวิชา พืชศาสตร์
ปีการศึกษา 2556

บทคัดย่อ

การชักนำต้นอ่อนยางพาราพันธุ์ดั้งเดิมผ่านกระบวนการไมโครคัตติ้ง โดยศึกษาผลของเปปโตน และซิลเวอร์ไนเตรท ต่อการสร้างยอดรวม จากการเพาะเลี้ยงข้อของต้นอ่อนยางพาราที่เก็บนอกหลอดทดลอง พบว่า การเติมเปปโตน (0-2 เปอร์เซ็นต์) ลงในอาหารสูตรชักนำการสร้างยอดรวม (Shoot induction medium: SIM) ซึ่งเป็นสูตรอาหาร Murashige and Skoog (MS) เติม 6-Benzyladenine (BA) 5 มิลลิกรัมต่อลิตร ผงถ่าน 0.5 เปอร์เซ็นต์ น้ำตาลซูโครส 3 เปอร์เซ็นต์ และผงวุ้น 0.75 เปอร์เซ็นต์ ให้เปอร์เซ็นต์การสร้างยอดรวม และจำนวนยอดต่อชิ้นส่วนลดลง เมื่อเปรียบเทียบกับที่อาหารที่ไม่เติมเปปโตน ลักษณะยอดที่เกิดขึ้นนั้นบางยอดมีขนาดเล็ก เหี่ยว และหลุดร่วง หลังจากการเพาะเลี้ยงเป็นเวลา 4 สัปดาห์ สำหรับการเติมสารละลายซิลเวอร์ไนเตรทความเข้มข้นระหว่าง 3-5 มิลลิกรัมต่อลิตร ส่งผลให้เกิดการสร้างยอดรวมได้ 100 เปอร์เซ็นต์ และจำนวนยอดเฉลี่ย 2 ยอดต่อชิ้นส่วน ไม่มีความแตกต่างทางสถิติระหว่างชุดทดลอง ($p < 0.05$) เมื่อลดความเข้มข้นของซิลเวอร์ไนเตรทลงเหลือ 0-2 มิลลิกรัมต่อลิตร พบว่า ความเข้มข้นของซิลเวอร์ไนเตรทที่ 1 มิลลิกรัมต่อลิตร เหมาะสมต่อการชักนำยอดรวมในยางพารา ซึ่งให้จำนวนยอดต่อชิ้นส่วนเพิ่มขึ้นเฉลี่ย 5 ยอดต่อชิ้นส่วน ยอดที่ได้มีสีเขียวเข้ม แข็งแรง ใบไม่หลุดร่วง ส่วนลักษณะยอดในอาหารที่ไม่เติมซิลเวอร์ไนเตรท มีสีเขียวอ่อน และบางยอดเกิดอาการเหี่ยว ใบหลุดร่วง หลังจากการเพาะเลี้ยงเป็นเวลา 4 สัปดาห์

สำหรับการชักนำต้นยางพาราพันธุ์ดั้งเดิมโดยผ่านกระบวนการสร้างโซมาติกเอ็มบริโอจากการเพาะเลี้ยงอับละอองเกสร พบว่า อาหารสูตรชักนำการสร้างแคลลัส (Callus induction medium) ซึ่งเป็นสูตรอาหาร MS เติม 2, 4 dichlorophenoxybenzoic acid (2, 4-D) 1 มิลลิกรัมต่อลิตร kinetin (KN) 1 มิลลิกรัมต่อลิตร α-naphthaleneacetic acid (NAA) 1 มิลลิกรัมต่อลิตร น้ำตาลซูโครส 3 เปอร์เซ็นต์ และผงวุ้น 0.75 เปอร์เซ็นต์ ส่งเสริมการสร้างแคลลัสได้ 100 เปอร์เซ็นต์หลังจาก

การเพาะเลี้ยงเป็นเวลา 4 สัปดาห์ อาหารสูตรที่เหมาะสมสำหรับการสร้างโซมาติกเอ็มบริโอคือสูตร MS เติม NAA 0.06 มิลลิกรัมต่อลิตร BA 0.03 มิลลิกรัมต่อลิตร น้ำตาลซูโครส 3 เปอร์เซ็นต์ และผงวุ้น 0.75 เปอร์เซ็นต์ โดยให้เปอร์เซ็นต์การสร้างโซมาติกเอ็มบริโอ 64 เปอร์เซ็นต์ และจำนวนโซมาติกเอ็มบริโอเฉลี่ย 5.13 โซมาติกเอ็มบริโอต่อชิ้นส่วน อย่างไรก็ตาม โซมาติกเอ็มบริโอดังกล่าวไม่สามารถพัฒนาเป็นต้นได้

จากการตรวจสอบปริมาณดีเอ็นเอของโซมาติกเอ็มบริโอเปรียบเทียบกับต้นแม่โดยเทคนิคโฟลไซโตเมทรี พบว่า โซมาติกเอ็มบริโอที่ชักนำได้มีปริมาณดีเอ็นเอเท่ากับต้นแม่ ส่วนการตรวจสอบความแปรปรวนทางพันธุกรรมของต้นยางพาราที่ได้จากกระบวนไมโครคัตติ้ง พบว่า ต้นยางพาราที่ชักนำได้มีรูปแบบของดีเอ็นที่เหมือนกันจากการตรวจสอบด้วยเครื่องหมาย RAPD ใช้ไพรเมอร์ 4 ชนิด (OPAD-01 OPAD-10 OPAD-12 และ OPB-17) และเครื่องหมาย SSR โดยใช้ไพรเมอร์ 3 ชนิด (*hmac4* *hmct1* และ *hmct5*) จากผลดังกล่าวแสดงให้เห็นว่าต้นยางพาราที่ได้จากกระบวนการนี้ไม่มีความแปรปรวนทางพันธุกรรม

Thesis Title	Propagation of Rubber (*Hevea brasiliensis* Muell. Arg.) through Somatic Embryogenesis and Assessment of Somaclonal Variation by Molecular Markers
Author	Miss Yupaporn Sirisom
Major Program	Plant Science
Academic Year	2013

ABSTRACT

Induction of plantlets through microcutting technique from early introduced clones rubber tree was the aim of this study. The effects of peptone and silver nitrate ($AgNO_3$) on *in vitro* shoot formation from cultured nodes of *Hevea brasiliensis* seedlings raised *ex vitro* were observed. The addition of peptone at concentration of 0-2% to the shoot induction medium (SIM) which consisted of Murashige and Skoog (MS) supplemented with 5 mg/l 6-Benzyladenine (BA), 0.5% activated charcoal, 3% sucrose and solidified with 0.75% agar was not successful in inducing multiple shoots. The number of shoots per explant decreased in comparison with the control treatment. Some shoots developed in the peptone-containing medium were small with senescence of leaves occurring after being cultured for 4 weeks. The addition of $AgNO_3$ at concentrations of 3-5 mg/l induced multiple shoot formation in all the explants at mean number of 2 shoots per explant. However, the average numbers of shoots per explant were not significantly different among all the concentrations tested. When the concentration of $AgNO_3$ decreased to 0-2 mg/l, the number of shoots per explant was improved. The best result in a mean number of shoots at 5 shoots per explant was achieved using a medium containing 1 mg/l $AgNO_3$. The shoots obtained on this medium had dark green leaves and vigorous growth, while those leaves of shoots obtained from the medium without $AgNO_3$ supplementation were pale green and senesced and fell down after 4 weeks of culture.

For somatic embryogenesis, the influence of plant growth regulators on somatic embryogenesis in anther culture of rubber tree was investigated. Callus was induced from immature anther on callus induction medium (CIM) which was MS medium

containing 1 mg/l 2, 4 dichlorophenoxybenzoic acid (2, 4-D), 1 mg/l kinetin (KN), 1 mg/l α-naphthaleneacetic acid (NAA) and 3% sucrose. This medium gave 100% callus induction after culture for 4 weeks. Optimum frequency of somatic embryogenesis at 64% and a number of somatic embryos (SEs) per explant at 5.13 SEs was achieved on MS supplemented with 0.06 mg/l NAA, 0.03 mg/l BA, 3% sucrose and 0.75% agar. However, these SEs could not develop into plantlet.

The assessment of the genetic instability of *in vitro* derived clones is considered to be a very useful and essential step in this study. The SEs derived from anther culture showed the same ploidy level as mother plants after evaluation by flow cytometry. For plantlet derived from microcutting technique, 5 primers (OPAD-01, OPAD-10, OPAD-12, OPB-17 and OPR-02) of RAPD and 3 primers (*hmac4*, *hmct1* and *hmct5*) of SSR marker gave the same profiles of DNA pattern. It was clear that somaclones obtained from our protocol were uniform and successfully used to assess genetic variations in micropropagated plants.

ACKNOWLEDGEMENT

I wish to express my deepest and sincere gratitude to my advisor, Associate Professor Dr. Sompong Te-chato for his valuable support, encouragement, supervision and useful suggestions throughout the course and correction of this research work.

I am grateful to thank the examining committees: Associate Professor Dr. Sayan Sdoodee from Department of Plant Science, Faculty of Natural Resources, Prince of Songkla University and Dr. Supawadee Ramasoot from Department of Science Education, Faculty of Science and Technology, Nakhon Si Thammarat Rajabhat University for their valuable comments and suggestions, which greatly contributed to a success of this thesis.

I also wish to thank all staff members of Plant Science Department, Faculty of Natural Resources, Prince of Songkla University for their helpful suggestions. For skillful technique assistance, I would like to thank all of the numbers of the Crop Biotechnology Laboratory who helped creating an enjoyable atmosphere to be working in and for their helpful in many countless ways throughout the years.

Financial supports have provided by the National Research University Project of Thailand's Office of the Higher Education Commission, the Graduate School of Prince of Songkla University. This research was partially supported by the Center of Excellence in Agricultural and Natural Resources Biotechnology, Postgraduate Education and Research Development Office, Commission on Higher Education, Ministry of Education.

Finally, I wish to express my deeply appreciation to my parents, aunt, sisters, brother, friends and my sweetheart for their love and support throughout my life.

Yupaporn Sirisom

CONTENTS

CONTENTS (continued)

LIST OF TABLES

LIST OF ILLUSTRATIONS

LIST OF ILLUSTRATIONS (continued)

LIST OF ILLUSTRATIONS (continued)

LIST OF ABBREVIATIONS AND SYMBOLS

AC	=	Activated charcoal
ANOVA	=	Analysis of variance
AFLP	=	Amplified fragment length polymorphism
BA	=	6-benzyladenine
bp	=	Base pair
CIM	=	Callus induction medium
CRD	=	Completely random design
CTAB	=	Cetyltrimethyl ammonium bromide
2,4-D	=	2,4-Dichlorophenoxyacetic acid
DMRT	=	Duncan's multiple range test
DNA	=	Deoxyribonucleic acid
dNTP	=	Deoxynucleotide triphosphate
EDTA	=	Ethylenediaminetetraacetic acid
GA_3	=	Gibberellic acid
IAA	=	Indole acetic acid
IBA	=	Indolebutyric acid
KN	=	Kinetin
M	=	Molar
ml	=	Milliliter
mm	=	Millimeter
mM	=	Millimolar
mg/l	=	Milligram per liter
MS	=	Murashige and Skoog (medium)
NAA	=	α- naphthalene acetic acid
$NaCl_2$	=	Sodium chloride
Na_2EDTA	=	Disodium ethylenediaminetetraacetate

LIST OF ABBREVIATIONS AND SYMBOLS (continued)

PVP	=	Polyvinyl pyrrolidone
RAPD	=	Random amplified polymorphic DNA
RFLP	=	Restriction fragment length polymorphism
RIM	=	Root induction medium
SIM	=	Shoot induction medium
SSCP	=	Single-strand conformation polymorphism
SSR	=	Simple sequence repeats
TDZ	=	Thidiazuron
TE	=	Tris EDTA
TAE	=	Tris-acetic acid-disodium ethylenediaminetetraacetic acetate
TBE	=	Tris-boric acid- disodium ethylenediaminetetraacetic acetate
Tris	=	Tris (hydroxymethyl) aminomethane
v/v	=	Volume per volume
w/v	=	Weight per volume
µl	=	Microlitter
µmol m^{-2} s^{-1}	=	Micromole per square meter per second
µM	=	Micromolar

LIST OF PAPERS AND PROCEEDINGS

1. Sirisom, Y. and Te-chato, S. 2012. The effect of peptone and silver nitrate on *in vitro* shoot formation in *Hevea brasiliensis* Muell Arg. International Journal of Agricultural Technology 8: 1509-1516.
2. Sirisom, Y. and Te-chato, S. 2013. Evaluation of anther-derived somatic embryos in *Hevea brasiliensis* Muell Arg. by flow cytometry. International Journal of Agricultural Technology 9: 713-720.
3. Sirisom, Y. and Te-chato, S. 2013. Assessment of somaclonal variation of rubber tree derived from microcutting by RAPD marker. The 12th National Toward AEC Under Climate Changes. Bangkok International Trade & Exhibition Centre, Bangkok, Thailand. 9-12 May 2013. pp. 24. (Oral presentation).
4. Sirisom, Y. and Te-chato, S. 2013. In vitro nodal culture of rubber tree and assessment of somaclonal variation by SSR marker. The 1st From Plant Science to AEC. Department of Plant Science, Faculty of Natural Resources, Prince of Songkla University, Hat Yai, Thailand. 13-14 August 2013. pp. 3. (Oral presentation).

SUMMARY OF CONTENTS

CHAPTER I

General Introduction

General Introduction

1.1 Background

Hevea brasiliensis Muell Arg., belonging to the Family Euphorbiaceae, is an economically important perennial tree crop grown in Thailand and Southeast Asia as the source of natural rubber. Natural rubber is considered to be a vital raw material in developed countries and is valued for its high-performance characteristics (Venkatachalam *et al*., 2006). Thailand is the world leading producer and exporter of rubber with production capacity of 3.1 – 3.2 million tons per year (88-90 percent of total production capacity exported to foreign markets). The country also has high potential for expanding production area and raising production capacity (Saengruksawong *et al*., 2012). *H. brasiliensis* is still propagated by grafting clonal axillary buds onto unselected seedlings to maintain intraclonal heterogeneity. In the past, the most common rootstocks used for rubber tree production in Thailand were seeds of any early introduced clones. At the present, a cultivar known as RRIM600 is the major cultivated variety of rubber plantations in Thailand (~70-75%). It is believed that almost all of the other early introduced clones have been gradually lost. A situation means that currently there is probably a high level of inbreeding clones used as rootstock (Nakkanong *et al*., 2008). Preliminary study of Khonglao (2006) on this situation indicated that there was higher vigorous rooting development of seedlings from early introduced clones than in RRIM600 seedlings. As the conventional method of propagation may lead to undesirable stock-scion interactions, the clonal propagation of *Hevea* by tissue culture is a relevant technique to a greater extent. The production of uniform individual plants is one of the main objectives for *in vitro* propagation of *Hevea* (Venkatachalam *et al*., 2006). Furthermore, many of the elite *Hevea* clones are susceptible to one or more of undesirable traits such as the physiological disorder and some important diseases especially white root disease. Thus, a reproducible plant regeneration system for

each genotype of *Hevea* through tissue culture is essential for further improvement programs.

1.2 Micropropagation of rubber tree

Nowadays, tissue culture technique is applied in many plant species including rubber tree. There have been several reports of rubber tree micropropagation using explants raised in different culture media selected according to the objective of the study. Most of the *in vitro* culture work in rubber tree is directed towards micropropagation through shoot tip culture, nodal cultures, and somatic embryogenesis. The first known work on *in vitro* culture of *Hevea* was carried out by Bouychou (1953) of the Institut Francais Caoutchouc, with the aim of using calli to obtain convenient material for the study of the laticeferous system (Nayanakantha and Seneviratne, 2007).

Micropropagation of rubber tree could be divided into two methods including microcutting and somatic embryogenesis. Microcutting technique begins by culturing axillary buds or cotyledonary nodes and induction of multiple shoots from them. Te-chato and Muangkaewngam (1992) induced multiple shoot from nodal culture of *in vitro* seedling of rubber, landrace cultivars, GT1 and PB5/51. Their results showed that MS (Murashige and Skoog, 1962) medium with BA (6-benzyladenine) alone at concentration of 4.5-5.63 mg/l gave 100% multiple shoot induction and the mean numbers of shoots per explant obtained from GT1, PB5/51 and landrace cultivar were 3.33, 3.00 and 3.00 shoots, respectively. Root induction was obtained from MS medium with IBA (Indolebutyric acid) in combination with NAA (α-Naphthaleneacetic acid) at equal concentration of 5 mg/l. Although micropropagation of clonal *Hevea* using axillary shoot proliferation has been achieved to a progressive level, there are a number of drawbacks in this system. A single plant could be produced from a single nodal explant and the plants that produced were

without a tap root leading to an undesirable in clonal tree propagation (Nayanakantha and Seneviratne, 2007).

For clonal improvement, most experiments use somatic embryogenesis. Somatic embryogenesis is one of the powerful tissue culture techniques for mass propagation of elite *Hevea* clones. The utilization of this system also opens up new protocol for production of mass number of uniform rootstock and for molecular farming through genetic transformation.

1.3 Somatic embryogenesis in rubber tree

Efficient plant regeneration through somatic embryogenesis is essential for mass propagation and crop improvement through transgenic approaches besides using this as a micropropagation system. *Hevea* somatic embryogenesis was firstly developed in China and Malaysia, using the anther as initial mother tissue explants (Venkatachalam *et al*., 2006). In *Hevea*, plant development via somatic embryogenesis was achieved from many explants including inner integument (Te-chato and Chartikul, 1993; Sushamakumari *et al*., 2000; Montoro *et al*., 2003; Lardet *et al*., 2007), immature anther (Wang *et al*., 1984; Jayasree *et al*., 1999; Hua *et al*., 2010), unpolinated ovules (Kouassi *et al*., 2008), root (Zhou *et al*., 2010) and embryo (Dickson *et al*., 2011).

Several factors such as the developmental stages and types of the explant types and concentrations of plant growth regulators and other growth substances, basal medium composition, light intensity, etc. appear to play important role in the induction and maintenance of somatic embryogenesis in many plants including *Hevea*. Jayasree *et al.* (1999) reported the optimized protocol for friable embryogenic callus induction, somatic embryogenesis and plant regeneration from the immature anthers. Optimum callus induction was obtained in modified MS medium supplemented with 2.0 mg/l 2, 4-D (2, 4-

Dichlorophenoxybenzoic acid) and 0.5 mg/l KN (Kinetin). Somatic embryo induction was found to be better with 0.7 mg/l KN and 0.2 mg/l NAA. Development of the embryos into plantlets was achieved on a hormone free medium. Cytological analysis revealed that all the plantlets tested were diploid. Subsequently, Hua *et al*. (2010) made attempts to regenerate plantlets through somatic embryogenesis from immature anther culture (clone CATAS 7-33-97 and CATAS 88-13). Optimum plantlet regeneration at 85% was obtained in modified MS medium supplemented with 4.5-13.5 µM 2, 4-D.

The success of embryoids and plant development via somatic embryogenesis was also achieved from anther wall-derived calli (Wang *et al*., 1984; Jayasree *et al*., 1999) which is somatic tissue. Thus, plantlets produced through this explant culture have been proved to be diploid and the same genetic composition as mother plant. For inner integument culture, Te-chato and Chartikul (1993) successfully induced embryogenic callus and plantlets regeneration from inner integument cultures of immature fruit (8 weeks after pollination) on modified MS medium supplemented with 2 mg/l 2, 4-D, 2 mg/l BA, 5-6% sucrose and adjusted pH to 5.6-5.8. Maturation and germination of these embryoids was promoted by excision of each embryoids and transfer to half strength liquid MS medium supplemented with 0.06 mg/l NAA and 0.03 mg/l BA overlaid on activated charcoal supplemented MS agar medium. Sushamakumari *et al*. (2000) developed a technique for somatic embryogenesis and plant regeneration using inner integument as explant. Their results shown that modified MS medium with 0.90 µM 2, 4-D, 2.68 µM NAA, 0.93 µM KN, 3% sucrose and 0.2% phytagel was suitable for callus induction. Somatic embryo induction was found on modified MS medium with 370 mg/l KH_2PO_4, 120 mg/l $MgSO_4.7H_2O$, 1.33 µM BA, 1.07 µM NAA, 3% sucrose and 0.2% phytagel and further development of the embryos into plantlets was achieved on this medium as described earlier. Montoro *et al*. (2003) and Lardet *et al*. (2007) have also induced embryogenic callus from integument culture (clone PB260) on MH medium supplemented with 4.5 µM 3,4-D

(3,4-Dichlorophenoxyacetic acid), 4.5 µM KN, 30 µM $AgNO_3$, 12 µM $CaCl_2$ and 234 mM sucrose. In addition, several researchers tried to induce somatic embryos from other explants of *Hevea*. For example, Zhou *et al*. (2010) successfully induced embryogenic callus from root culture derived from *in vitro* seedling of clone Reyan 87-6-62. Somatic embryo was developed on modified MS medium supplemented with 1 mg/l KN, 0.2 mg/l BA and plantlets could be germinated on liquidified MS medium with 0.5 mg/l KN, 0.2 mg/l IAA (Indoleacetic acid) and 0.3 mg/l GA_3 (Gibberellic acid) at 11.8% after culturing for 8 weeks. However, an efficient protocol for the large scale micropropagation of elite *Hevea* clones has not yet been developed (Nayanakantha and Seneviratne, 2007).

1.4 The application of molecular markers

At present, the development of methods to assess the genetic instability of *in vitro* plants is highly valuable such as chromosomal analysis, flow cytometry, and DNA fingerprinting by molecular markers. Different molecular analytical techniques have been being used to point out somaclonal variation in tissue culture and in regenerants of several plants. RAPD (Randomly amplified polymorphic DNA) and SSR (Simple sequence repeat) markers are used widely in studying the genetic variability in micropropagated plant such as kiwifruit (Palombi and Damiano, 2002), banana (Sheidai *et al*., 2008), potato (Karacsonyi *et al*., 2011), *Silybum marianum* (Mahmood *et al*., 2010).

RAPD marker is technically simple and cheap. RAPD analysis using PCR (Polymerase chain reactions) in association with short primers of arbitrary sequence has been demonstrated to be sensitive in detecting variation among individuals. PCR products are separated on agarose gel and detected by staining with ethidium bromide (Williams *et al*., 1990). Most RAPD fragments are inherited as dominant markers. A fragment is observed in the homozygous. The advantages of this technique are a large number of samples can be

quickly and economically analyzed using only micro-quantities of material (Sheidai *et al*., 2008). DNA fragment profiles have been employed to analyze the genetic relationships of plant species (Ayana *et al*., 2002). RAPD analysis is based on the presence or absence of polymorphisms in individuals or groups of individuals (Tingey and Tufo, 1993). The markers have been employed in the analysis of genetic variation of *in vitro* plant. For example, Barakat and El-Sammak (2011) determined characterization of mutants in Baby's breath *Gypsophila paniculata* after gamma irradiation. Ehsanpour *et al*. (2007) also analyzed somaclonal variation among potato callus induced by UV-C radiation. In addition, Sheidai *et al*. (2008) investigated somaclonal variation in banana derived from long time-period of subculture.

SSRs or microsatellites are tandem repeats of 1-6 nucleotides and are present in all eukaryotic genomes. The method is very accurate and reproducible, being able to detect any variation within repeats number, determined by addition or deletion of repeat units, or by point mutations (Ehsanpour *et al*., 2007). Their polymorphism is revealed by the PCR using flanking primers that generate co-dominant markers. SSR marker is considered suitable markers for genetic studies. They have the advantage of combining co-dominance and high polymorphism plus abundance and uniform dispersion in plant genomes. In plants, SSR markers have been successfully applied to a variety of questions, including the construction of genetic maps, assessment of genetic diversity, cultivar identification and pedigree studies (Roy *et al*., 2004). Jin *et al*. (2008) detected somaclonal variation of regenerated cotton plants via somatic embryogenesis by the use of this technique. Karacsonyi *et al*. (2011) also use this technique for analyze somaclonal variation in potato plantlets regenerated from callus.

Several methods of identifying molecular markers have been used with *H. brasiliensis* including assessment of genetic diversity in wild and cultivated clones by RFLP (Restriction fragment length polymorphisms) (Besse *et al*., 1994), mitochondrial DNA (Luo

et al., 1995) and RAPD (Cesar *et al.,* 2006), evaluation of the genetic relatedness of wild and cultivated *Hevea* accessions with SSCP (Single-strand conformation polymorphism) (Lekawipat *et al*., 2003) and microsatellite or SSR (Roy *et al*., 2004; Saha *et al*., 2005) and identification of dwarf genome by RAPD marker (Venkatachalam *et al*., 2004). Nakkanong *et al*. (2008) analyzed 53 early introduced clones of rubber tree collected from different areas in southern Thailand. The assessment was performed using RAPD and microsatellite markers. Their results showed that eight primers (OPB-17, OPN-16, OPR-02, OPR-11, OPZ-04, OPAD-01, OPAD-10 and OPAD-12) were chosen for genetic variation analysis in 87 individual plants. Four microsatellite primer pairs (*hmac*4, *hmct*1, *hmct*5 and *hmac*5) produced a total of 44 amplified fragments with an average of 14.67 fragments per primer, of which 37 were polymorphic (84.09%) while *hmac5* produced only monomorphic fragments. So far, assessment of genetic instability in micropropagation of *Hevea* has been reported using only chromosome analysis (Jayashree, 1999).

Therefore, our attention in the present study was focused on the micropropagation of early introduced clones of rubber tree using different explants (node, shoot tip and immature anther). Moreover, Flow cytometry, RAPD and SSR analysis were used to confirm the uniformity of regenerated plantlets before cultivation in the field and further using as rootstock commercially.

1.5 Objectives

1. To develop culture medium for plantlet regeneration of early introduced clones of rubber tree through microcutting technique

2. To develop culture medium for callus induction and plantlet regeneration from anther culture of early introduced clones of rubber tree through somatic embryogenesis

3. To assess genetic instability of *in vitro* plantlets by some molecular markers

4. To conserve early introduced clones of rubber tree *in vitro*

CHAPTER II

Micropropagation of Rubber Tree through Microcutting

Experiment I

Effect of Peptone and Silver Nitrate on Multiple Shoot Formation

Introduction

Micropropagation of rubber tree could be divided into two methods including microcutting and somatic embryogenesis. In order to propagate true-to-type clones, the microcutting technique is always used. This technique begins by culturing axillary buds or cotyledonary nodes and then inducing plantlets from them. Te-chato and Muangkaewngam (1992) induced multiple shoot from nodal culture of *in vitro* seedling of rubber cultivars, local, GT1 and PB5/51. Their results showed that MS medium with 4.5-5.63 mg/l BA gave 100% shoot induction and the mean numbers of shoots per explant obtained from GT1, PB5/51 and local cultivar were 3.33, 3.00 and 3.00, respectively. Root induction was obtained from MS medium with IBA in combination with NAA at equal concentration of 5 mg/l. In preliminary studied, we tried to use the medium according to the researchers as described earlier. Pale green with senescence of the leaves were observed after 4 weeks of culture. Therefore, the used of some complex compounds adding in the medium might be improved shoot formation in rubber tree tissue culture without those syndromes.

There have been several reports on using peptone to improve multiple shoot formation in tissue culture of crop species, for instance orchids (Seeni and Latha, 1992; Chen and Chang, 2002) and avocado (Nhut *et al*., 2008). Silver nitrate ($AgNO_3$) has also been shown to be effective in improving plantlet regeneration during somatic embryogenesis in a number of crop species, including brussels sprouts (Williams *et al*., 1990), cassava (Zhang *et al*., 2001), *Paspalum scrobiculatum* L. (Vikrant and Rashid, 2002), *Ziziphus jujuba* Mill. (Feng *et al*., 2010), *Bixa orellana* L. (Parimalan *et al*., 2010) and turnip (Cogbill *et al.,* 2010). However, the uses of peptone and silver nitrate have not yet been reported in rubber tree. Therefore, in the present study, we report on the *in vitro* shoot multiplication of rubber tree using peptone and silver nitrate in order to mass propagation of uniform plantlets from this tree species.

Materials and methods

Plant materials

One-month-old seedlings of early introduced clones of rubber tree grown naturally at Prince of Songkla University, Hatyai campus, Songkhla province, Thailand, were used in this experiment. The early introduced rubber clones were recognized by their big trunk indicating an age of more than 50 years and their random location outside established rubber plantation areas (Nakkanong *et al.,* 2008).

Effect of peptone and silver nitrate on multiple shoot formation

To study the effect of peptone and silver nitrate on multiple shoot formation shoot induction medium (SIM; MS medium supplemented with 5 mg/l BA, 3% sucrose, 0.05% activated charcoal) as described by Te-chato and Muangkaewngam (1992) was used in this experiment. The pH of culture medium was adjusted to 5.7 with 0.1 N HCl or KOH before adding 0.75% agar and autoclaving at 1.05 kg/cm^2, 121ºC for 15 minutes. The cultures were maintained at 28±0.5° C under fluorescent bulbs at 12.5 µmol/m^2/s for a 14 hour photoperiod. The shoot tips or nodes were cultured on SIM under the conditions as specified above for 4 weeks. The cultures were routinely subcultured at 4 week intervals to induce multiple shoot formation. After being cultured for 4 weeks, the micro-shoots obtained on the SIM were individually excised and transferred to SIM, supplemented with various concentrations of peptone (0, 1, 1.5, and 2%) or silver nitrate (0, 3, 4 and 5 mg/l). After being cultured for further 6 weeks, the extent of multiple shoot formation and the number of shoots per explant was recorded.

Improved multiple shoots formation by silver nitrate

The micro-shoots cultured on the SIM were individually excised and transferred to SIM supplemented with various concentrations of silver nitrate all of which

were lower than in previous experiments (0, 0.5, 1 and 2 mg/l). The cultures were maintained under the same conditions as described above. After 6 weeks of culture, the extent of multiple shoot formation and the number of shoots per explant were recorded. Six-week-old multiple shoots derived from the SIM were transferred to a growing medium based on solidified MS medium supplemented with 1 mg/l silver nitrate and 0.05% activated charcoal but without the addition of a plant growth regulator to encourage shoot elongation. After 4 weeks of culture, each elongated shoot was excised and transferred to a half-strength MS medium supplemented with 5 mg/l IBA, 1 mg/l silver nitrate, 3% sucrose, 0.05% activated charcoal and 0.75% agar for root induction. Complete plantlets were then hardened and transferred to a greenhouse. The data were statistically analyzed using completely randomized design (CRD) and the means among the treatments were separated by Dancan's multiple range test (DMRT).

Results and Discussion

The use of the microcutting technique, the shoots and nodes of seedlings can be successfully used for *in vitro* multiplication on the SIM as earlier described by Te-chato and Muangkaewngam (1992). In exceptional cases, the multiple shoots produced from a node were more vigorous than those produced from a shoot tip culture. In our results, peptone and silver nitrate at concentrations of 3 mg/l or higher had severely affected the formation of multiple shoots on cultured shoot tips of rubber tree. Peptone at all concentration tested reduced the average number of shoots per explant when compared with control treatment (culture medium without peptone). The formation of multiple shoots was detected after 3 weeks of culture on peptone containing SIM. Multiple shoot formation frequency ranging from 93 to 100% obtained from culturing the explants on SIM supplemented with all concentrations of peptone without significant difference. Significant difference was obtained between the control medium and the peptone-supplemented media

(Table1). Addition of peptone at all concentrations to culture medium gave far lower number of shoots produced from single shoot tip. There was non significant difference among concentration of peptone tested on a number of shoot formation. A maximum number of shoots produced from single shoot tip cultured in all concentrations of peptone containing medium were never exceed 3 shoots. In addition, some shoots were small and wilted after 4 weeks of being cultured (Figure 1). Peptone has been reported to use as the source of carbon and nitrogen for plant tissue culture (Nhut *et al*., 2008) and some reports have shown a positive effect on the growth of explants, including embryo production in *Oncidium* (Chen and Chang, 2002) and shoot multiplication in avocado (Nhut *et al*., 2008). However, in the present study, the number of multiple shoots produced in the presence of peptone after being cultured for 4 weeks on SIM was small with senescence of the leaves, suggesting that peptone might inhibit shoot formation in this plant.

The explants cultured in SIM supplemented with silver nitrate formed multiple shoots at all concentrations. The number of shoots was approximately three shoots per cultured single shoot and not significantly different among the different concentrations tested (Table 1). Significant difference was not seen between the number of shoots produced in the control medium and the silver nitrate-containing media. However, the number of shoots obtained from silver nitrate containing medium was less than that in the control medium. After being cultured for 4 weeks, the shoots had dark green leaves and produced roots in a similar manner to shoots cultured in SIM without the supplement of silver nitrate. (Figure 2a, b). The mode of action of silver nitrate in plant tissue culture is assumed to be associated with the physiological effects of ethylene. Silver ions act as a competitive inhibitor of ethylene action rather than inhibiting ethylene synthesis (Zhang *et al*., 2001). Many reports have demonstrated the positive effect of silver nitrate on plant tissue culture (Zhang *et al*., 2001; Vikrant and Rashid, 2002; Feng *et al*., 2010; Parimalan *et al*.,

2010; Cogbill *et al*., 2010). In the present study, the addition of silver nitrate in the induction medium at concentrations ranging from 0.5 to 2 mg/l was successful in promoting shoot multiplication in all the explants with an average numbers of 5 shoots per explant. In addition, silver nitrate provides silver ions which may interact with polyamines, leading to the promotion of organogenesis and embryogenesis (Zhang *et al*., 2001). Normally, ethylene inhibits S-adenosyl methionine (*SAM*) decarboxylase, which in turn promotes polyamine (Parimalan *et al*., 2010). Thus, the present study suggests that it is possible to improve the frequency of shoot organogenesis in *H. brasiliensis* by supplementing silver nitrate in SIM.

These findings suggest that silver nitrate plays a more significant role in shoot formation than peptone. However, the optimum concentration noted in this phase of the study was not actually optimal for producing the maximum number of shoots, since the control medium without silver nitrate resulted in the production of the larger number of shoots. After the concentrations of silver nitrate were minimized to 1-2 mg/l an average of 5 shoots per explants were obtained. However, there was non significant difference among the concentrations tested (Table 2). The shoots cultured on SIM containing silver nitrate were dark green and exhibited vigorous growth (Figure 3b-d) while those cultured on the control medium without silver nitrate were pale green and exhibited senescence of the leaves after 4 weeks of culture (Figure 3a). Silver nitrate at a concentration of 1 mg/l proved to be an optimal for multiple shoot induction.

Elongation of shoots was carried out by transferring small clusters of multiple shoots developed in SIM to solidified MS medium without plant growth regulators in the presence of 1 mg/l silver nitrate and 0.05% activated charcoal. After 4 weeks of culture roots were formed simultaneously (Figure 4a). In case of non-rooted shoots, elongated single shoot at 2–3 cm long was excised from the multiple shoot clump and transferred to solidified MS medium supplemented with 5 mg/l IBA, 5 mg/l NAA, 1 mg/l silver nitrate, 3% sucrose

and 0.05% activated charcoal. The shoots developed roots at 100% with a mean number of 7.33±3.56 roots per shoot (Figure 4b; Figure 5) after culture for 4 weeks.

Table 1 Effect of peptone or silver nitrate on multiple shoot formation from culturing shoot tip explants on SIM for 6 weeks.

Concentration of substance	Multiple shoot induction (Mean %)	Average number of shoots/explant
Peptone (%)		
0	100	3.40 ± 0.55a
1	93.33	1.60 ± 0.55b
1.5	100	1.40 ± 0.55b
2	93.33	1.60±0.55b
F-test	ns	*
C.V. (%)	0	13.04
Silver nitrate (mg/l)		
0	100	3.40 ± 0.14
3	100	3.00 ± 0.84
4	100	2.60 ± 0.89
5	100	2.20 ± 0.45
F-test	ns	ns
C.V. (%)	0	14.22

*Significant different at $p < 0.05$

Mean values followed by the same letter(s) within a column are not significantly different ($P < 0.05$) by DMRT; ± indicates standard deviation

Table 2 Effect of various concentrations of silver nitrate on average number of shoots per explant on SIM supplemented with 5 mg/l BA, 3% sucrose, and 0.05% activated charcoal after being cultured for 6 weeks.

Concentration of silver nitrate (mg/l)	Multiple shoots induction (%)	Average number of shoots/explants
0	100	3.50 ± 1.35
0.5	100	4.20 ± 2.68
1.0	100	5.60 ± 3.36
2.0	100	5.29 ± 1.29
F-test	ns	ns
C.V. (%)	0	27.11

± indicates standard deviation

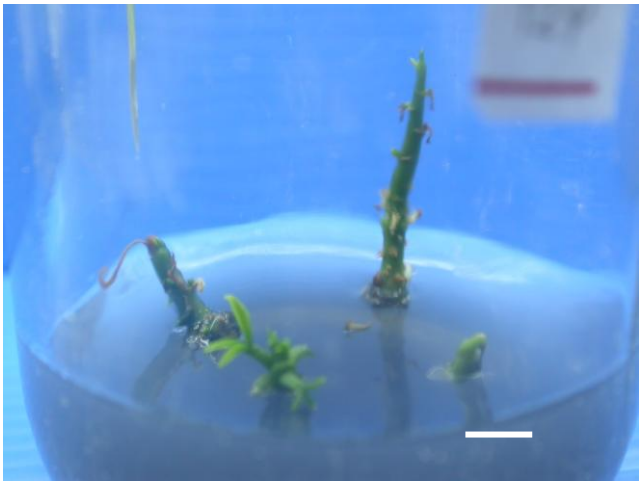

Figure 1 Small wilted shoots on SIM medium supplemented with 2% peptone after culturing for 4 weeks (bar = 0.5 cm).

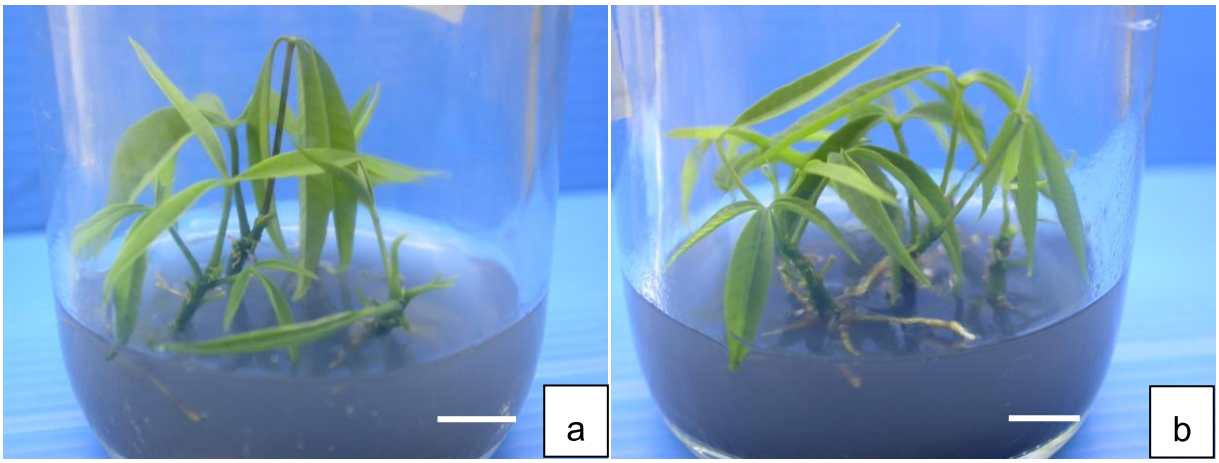

Figure 2 The shoots had dark green leaves and produced roots in a similar manner to shoots cultured in SIM without the supplement of silver nitrate (a) and SIM with 5 mg/l silver nitrate (b) after culturing for 4-6 weeks (bar=0.25 cm).

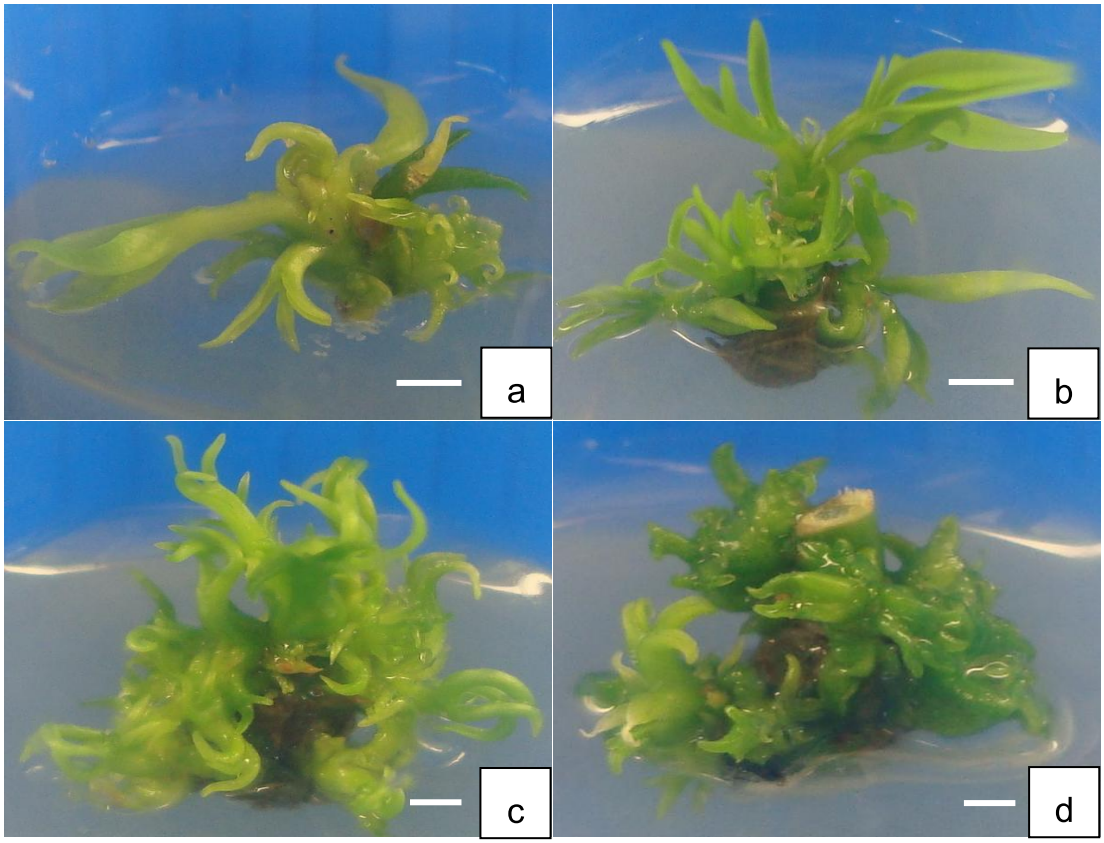

Figure 3 Multiple shoots formation on SIM supplemented with various concentration of silver nitrate after cultured for 4 weeks; (a) control treatment without silver nitrate, (b) 0.5 mg/l silver nitrate, (c) 1 mg/l silver nitrate and (d) 2 mg/l silver nitrate (bar = 0.5 cm).

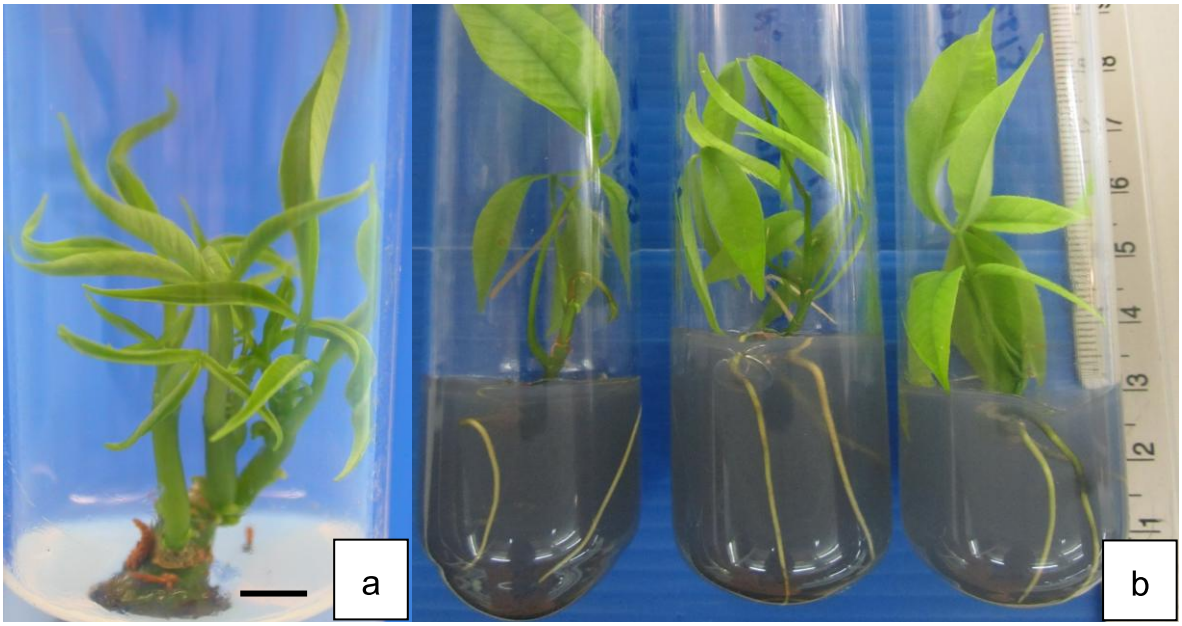

Figure 4 Elongation shoots on MS medium without plant growth regulators for 4 weeks (a) (bar = 0.5 cm) and root induction on MS medium supplemented with 5 mg/l IBA, 5 mg/l NAA, 3% sucrose and 0.75% agar (b).

Figure 5 Complete plantlets after cultured on MS medium supplemented with 5 mg/l IBA, 5 mg/l NAA, 3% sucrose and 0.75% agar for 4 weeks.

CHAPTER III

Micropropagation of Rubber Tree through Somatic Embryogenesis

Experiment I

Effect of Plant Growth Regulators on Somatic Embryo (SE) Formation

Introduction

Somatic embryogenesis is one of the powerful tissue culture techniques for mass propagation of elite *Hevea* clones. The utilization of this system also opens up new avenues for production of mass number of uniform rootstock and for molecular farming through genetic transformation. *Hevea* somatic embryogenesis was firstly developed in China and Malaysia, using the anther as initial mother tissue explants (Venkatachalam *et al*., 2007). Plant development via somatic embryogenesis in *Hevea* was achieved from many explants including inner integument (Te-chato and Chartikul, 1993; Sushamakumari *et al*., 2000; Montoro *et al*., 2003; Lardet et al., 2007) axillary buds (Mendanha *et al*., 1998) immature anther (Wang *et al*., 1984; Jayasree *et al*., 1999) unpollinated ovules (Kouassi *et al*., 2008) and root (Zhou *et al*., 2010). Anthers culture offers the possibility of homozygous haploid lines production. However, the long juvenile phase and the requirement of many generations in breeding program make this approach impractical. *In vitro* approaches to induce haploids in *Hevea* have only limited success in comparison with other plant species (Venkatachalam *et al*., 2006). The success of embryoids and plant development via somatic embryogenesis was achieved from anther wall-derived calli (Wang *et al*., 1984; Jayasree *et al*., 1999) which is somatic tissue. Thus, plantlets produced through this explants culture are diploid and the same as mother plant. So far, assessment of genetic instability in micropropagation of *Hevea* has been reported using only chromosome analysis (Jayashree, 1999). However, assessment of those plantlets needs to be performed.

Materials and methods

Plant materials

The male flowers (3-3.5 mm in length; Figure 6) from panicle inflorescence collected from early introduced clones of rubber tree grown naturally at Prince of Songkla University, Hatyai campus, Songkhla province, Thailand were used as mother plants for the source of anther in the callus initiation experiment. The flowers were contained uninucleate microspores confirmed by periodically determined under compound microscope (Figure 7)

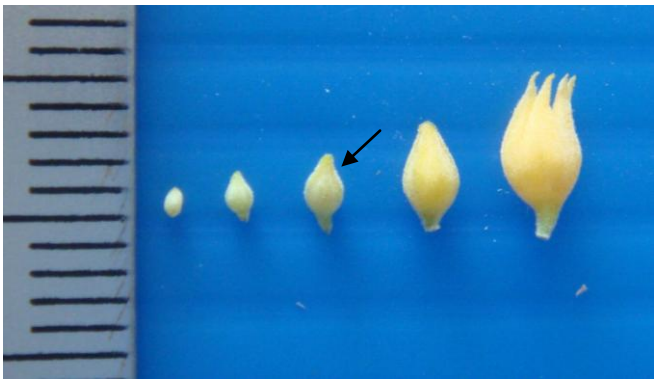

Figure 6 Different sizes of male flowers from panicle inflorescence of early introduced clone of rubber tree grown naturally at Hat Yai campus of Prince of Songkla University, Songkhla province, Thailand. (The arrow indicate male flower used in this experiment)

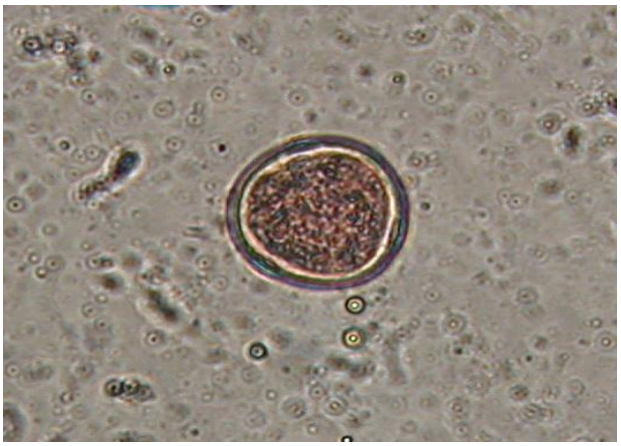

Figure 7 Uninucleate stage of anther from male flowers (3-3.5 mm in length) after observation under microscope.

Callus induction

The immature anthers were excised from sterilized male flowers and cultured on a callus induction medium (CIM) which was MS medium supplemented with 1 mg/l 2, 4-D, 1 mg/l KN, 1 mg/l NAA and 3% sucrose (Te-chato *et al*., 2002). The pH of culture medium was adjusted to 5.7 with 0.1 N HCl or KOH before adding 0.75% agar and autoclaving at 1.05 kg/cm^2, 121ºC for 15 minutes. The cultures were maintained at 28±0.5^{o}C under fluorescent bulbs at 12.5 µmol/m^2/s for a 14 hour photoperiod. The cultures were routinely subcultured at 4 week intervals to induce and proliferation of callus.

Effect of plant growth regulators on SE induction

The immature anthers-derived calli on CIM under the conditions specified above for 8 weeks were used in this study. The calli (100 mg FW) were transferred to somatic embryos (SEs) induction medium which was MS medium supplemented with BA at different concentrations of (0-1 mg/l) or 2, 4-D (0-1 mg/l) or 0.06 mg/l NAA and 0.03 mg/l BA. All culture media were supplemented with 3% sucrose. The pH of culture media was adjusted to 5.7 with 0.1 N HCl or KOH before adding 0.75% agar and autoclaving at 1.05 kg/cm^2, 121ºC for 15 minutes. The cultures were maintained at 28±0.5^{o}C under fluorescent bulbs at 12.5 µmol/m^2/s for a 14 hour photoperiod. The cultures were routinely subcultured at 4 week intervals to induce SE formation. After culture for 8 weeks, the percentage of SE formation and the number of SEs per explant were recorded.

Results and Discussion

Extensive experiments were carried out by several researchers to enhance the frequency of SE induction and plant regeneration in *Hevea*. In this study, immature anther-derived calli were achieved on CIM medium. Friable callus and fast growing were observed on this medium. After culture the callus on SE induction medium with different

concentrations of plant growth regulators for 8 weeks, SE was developed from peripheral cells of the calluses in all culture media. The frequency of somatic embryogenesis and average number of SEs per explant increased when increasing the concentration of BA or 2, 4-D (Table 3). In the absence of plant growth regulators, callus failed to initiate SEs and died shortly after being cultured for 2 weeks (Figure 8). However, SEs obtained from culture media containing BA or 2, 4-D were abnormal and ceased to develop at torpedo stage (Figure 9a-b). Although MS medium containing 2, 4-D in combination with BA gave callus formation but those calli could not develop into SEs (data not shown). Induction of SEs was found to be better in culture medium with 0.06 mg/l NAA and 0.03 mg/l BA. Percentage and number of SE induction were recorded to be 64.00 ± 8.94 and 5.13 ± 2.03 SEs/ explants, respectively (Table 3). Those SEs could develop into the cotyledonary stage and tend to germinate into plantlet (Figure 10). Some SEs was developed from browning callus after cultured on SEs induction medium for 8 weeks (Figure 11).

Several factors such as the developmental stages, types of explant, plant growth regulators, basal medium composition, light intensity, etc. appear to play important role in the induction of somatic embryogenesis in many plants including *Hevea*. In the present study, modified MS medium supplemented with BA or 2, 4-D alone could induce SEs but those SEs did not developed into the mature SEs. Hua *et al*. (2010) observed that MS-based plant regeneration medium with 4.5 or 9.0 μM 2, 4-D gave the highest rate of plant regeneration through SEs from root culture of *Hevea* (CATAS 7-33-97 and CATAS 88-13) which was difference from our results. In addition, several researchers reported that MS medium containing 0.06 mg/l NAA and 0.03 mg/l BA gave the suitable embryos induction and plant conversion in many plants including mangosteen, pawa (*Garcinia speciosa* Wall.) and somkhag (*G. atroviridis* Griff.) (Te-chato, 1997). Therefore, in this study, we tried to modify this medium for SEs induction in *Hevea* from anther-derived callus. The

results showed that MS medium containing 0.06 mg/l NAA and 0.03 mg/l BA gave the better result in SE induction percentage and a number of SEs per explant than other culture media. The low level of auxin and cytokinin in the SE induction medium was sometimes responsible for the development of embryos directly from the explants, implying that they did not develop through callus formation (Kouassi *et al*., 2008). However, our results showed that embryos were induced indirectly through callus induction.

Table 3 Effect of MS medium supplemented with different concentrations of BA or 2, 4-D or NAA on SE formation from anther-derived callus of early introduced clones of rubber tree grown naturally at Prince of Songkla University, Hatyai campus, Songkhla province, Thailand, for 8 weeks.

Concentrations of PGR	SE formation (%)	Average no. of SEs/explant
BA (mg/l)		
0	0c	0c
0.1	33.33±14.83b	1.67±1.09b
0.25	40.00±11.40b	2.00±1.48b
0.50	73.33±8.37a	3.67±1.30a
1.0	86.67±8.94a	4.33±3.67a
F-test	*	*
C.V. (%)	28.87	12.78
2,4-D (mg/l)		
0	0c	0c
0.25	48.00±11.40b	2.40±0.78b
0.50	56.00±13.04b	2.80±0.67b
0.75	52.00±8.94b	2.60±1.24b
1.0	84.00±10.95a	4.20±1.78a
F-test	*	*
C.V. (%)	26.16	14.59
0.06 mg/l NAA + 0.03 mg/l BA		
	64.00±8.94	5.13±2.03

*Significant different at $p < 0.05$

Mean values followed by the same letter(s) within a column are not significantly different ($P < 0.05$) by DMRT; ± indicates standard deviation

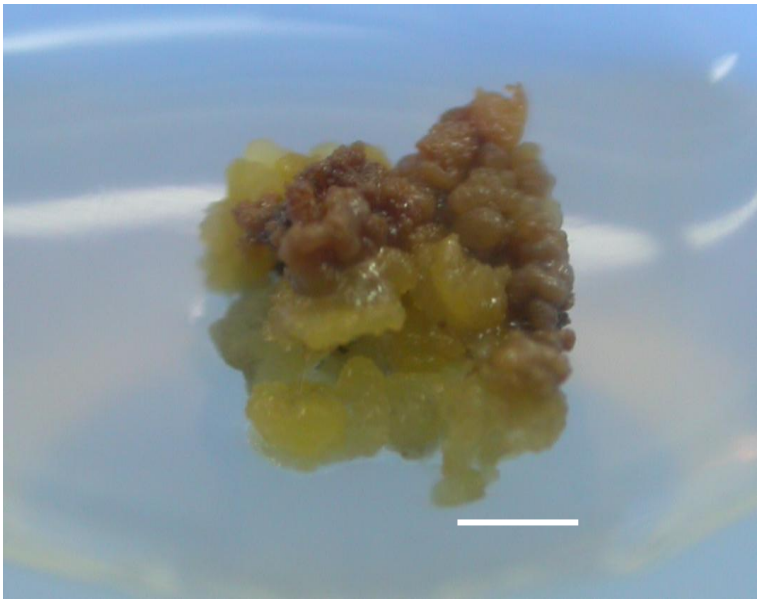

Figure 8 Browning callus on MS medium without plant growth regulators after culturing for 2 weeks (bar=0.25 cm).

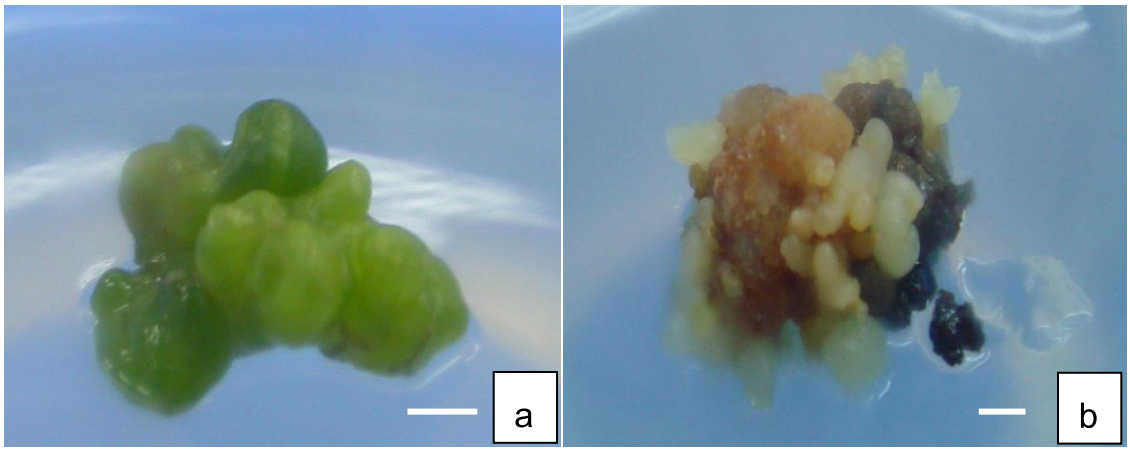

Figure 9 Greenish color SEs on MS medium with 1 mg/l BA (bar=0.5 cm) (a) and yellowish color SEs on MS medium with 1 mg/l 2, 4-D after culturing for 8 weeks (bar=0.25 cm) (b).

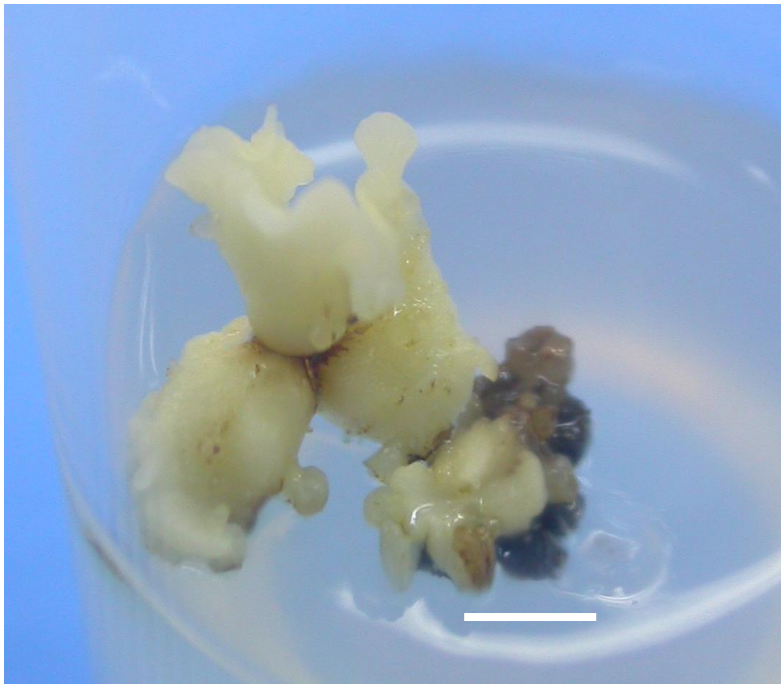

Figure 10 Well developed SEs on MS medium with 0.06 mg/l NAA and 0.03 mg/l BA after culturing for 8 weeks (bar = 0.25 cm).

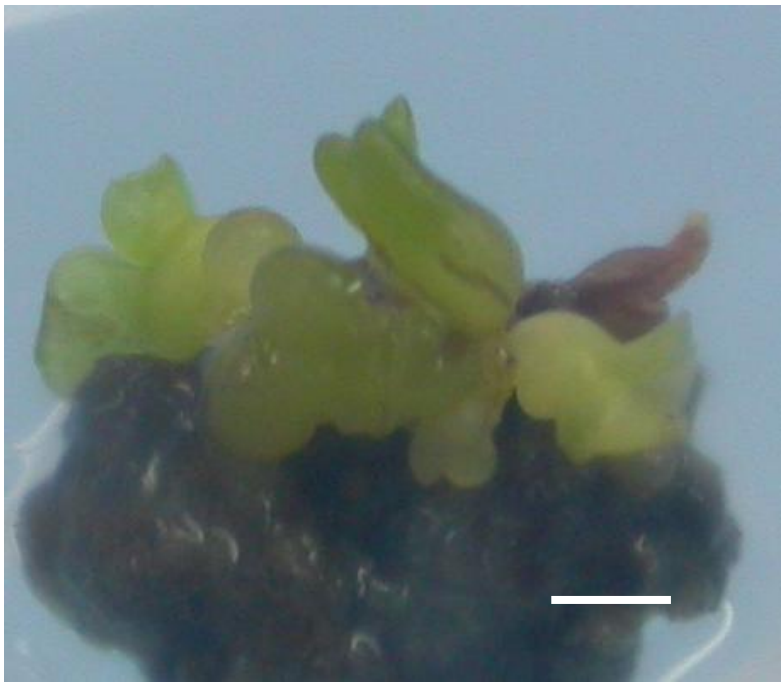

Figure 11 SEs regenerated from browning callus on MS medium with 0.06 mg/l NAA and 0.03 mg/l BA after culturing for 8 weeks (bar = 0.25 cm).

CHAPTER IV

Assessment of Genetic Instability

Experiment I

Determination of Ploidy Levels of Anther-derived SEs by Flow Cytometry Analysis

Introduction

Measurement of cellular DNA content or ploidy levels and the analysis of the cell cycle can be performed by flow cytometry. The principle is that the stained material has incorporated an amount of dye proportional to the amount of DNA. The stained material is then measured in the flow cytometer and the emitted fluorescent signal yields an electronic pulse with a height (amplitude) proportional to the total fluorescence emission from the cell. Thereafter, such fluorescence data are considered a measurement of the cellular DNA content (Nunez, 2001). The use of flow cytometry to detect DNA contents in plant tissue, cell, and organ culture methods has been increasing. The most common genetic variation observed in plant tissue culture is increase in ploidy level, and flow cytometry has been effective in identifying this type of change. Flow cytometry has also been used to evaluate ploidy stability in bahiagrass (*Paspalum notatum*) (Gondo *et al*., 2005), somatic embryogenesis-derived *Passiflora cincinnata* Mast. plants (Pinto *et al*., 2010) and analyzing of DNA content of somatic embryos, shoots, and calli of cactus (Lema-Ruminska, 2011). So far, only chromosome analysis was reported for evaluation the genetic instability of anther-derived plantlets (Jayashree, 1999). So the aim of this study was to evaluate the ploidy level of somatic embryos (SEs) derived from anther culture in *H. brasiliensis* by flow cytometry, which simple, fast and efficient.

Materials and methods

Plant materials

Well-developed SEs derived from anther culture on SE induction medium (MS + 0.06 mg/l NAA + 0.03 mg/l BA + 3% sucrose + 0.75% agar) for 2 months were used to determine DNA content in comparison with the mother plant. The mother plant used in this study was the early introduced rubber clones which recognized by their big trunk

indicating an age of more than 50 years and their random location outside established rubber plantation areas (Nakkanong *et al.,* 2008).

Flow cytometry analysis

Flow cytometry was performed to estimate the ploidy level of those SEs according to the method described by Ishigaki *et al*. (2009). In brief, approximately 0.5 cm^2 of individual SE derived from anthers culture or young leaf tissue from the mother plants were cut and placed on 90-mm Petri dishes. These explant pieces were soaked with 1 ml of an extraction buffer (50 mol/l Tris–HCl, 0.5% polyvinylpyrrolidone, 0.01% Triton-X, 0.63% sodium sulfite, pH 7.5) for 5 minutes and chopped with a sharp razor blade. After filtering chopping tissue through 50 µm nylon mesh, 100 µl of 0.1% propidium iodide (PI) solution was added to the nuclear suspension and incubated for at least 5 min at room temperature to stain the nuclei. The fluorescent intensities of each nuclear suspension were measured by an EPICS XL, equipped with a 488 nm argon laser with a long path filter (Beckman Coulter, Tokyo, Japan). The DNA content demonstrated as histogram peak of individual SE and leaf of mother plants was compared.

Results and Discussion

To evaluate ploidy stability of regenerants or regeneration SE via somatic embryogenesis flow cytometry was employed. The DNA content of nuclei from SEs was determined. The use of flow cytometry for nuclear DNA content analysis is an excellent alternative for assessing clonal fidelity (Jin *et al*., 2008). It was employed to estimate the ploidy level of regenerated plantlets from SEs due to the accuracy, short time require and large number of samples done in comparison with chromosome counting technique (Ishigaki *et al*., 2009). Flow cytometry is regarded as the most accurate tool for ploidy estimation, whereas traditional cytological analysis takes more time and labor intensive

(Gu *et al*., 2005; Yang *et al*., 2006). In our study, determination the ploidy level of those SEs (Figure 12b-i) by flow cytometry technique, showed the same peak as mother plant (Figure 12a) at the specific chanel 450-500. Previous cytological observation in *Hevea* indicated that regenerated plantlets induced from anther culture were diploid (Wang *et al*., 1984; Jayasree *et al*., 1999), suggesting that callus might be developed from parenchyma cells at the cut end and apex end in the septum and wall. The pollen grains in the anther disintegrated and died shortly after being cultured (Wang *et al*., 1984). Genetic performance also proved that anther SEs could originate from somatic cells or tissues of the anther. Uniformity in ploidy level has also been reported in *Vitis vinifera* L. regenerated via somatic embryogenesis induced in immature zygotic embryos. Regenerants analyzed by flow cytometry showed the same ploidy level as the donor plants maintained in the field (Yang *et al*., 2008). However, some reports showed variation of DNA content among micropropagation system. For example, Lema-Ruminska (2011) analyzed DNA content of nuclei isolated from *in vitro* grown somatic embryos, shoots and calli, as well as mammillae of *in vivo* grown shoots of cactus. Their results showed variation of DNA contents (2C up to 32C) among those explants. Based on the results of this study, it is suggested that rubber tree regenerated via somatic embryogenesis maintained true-to-type.

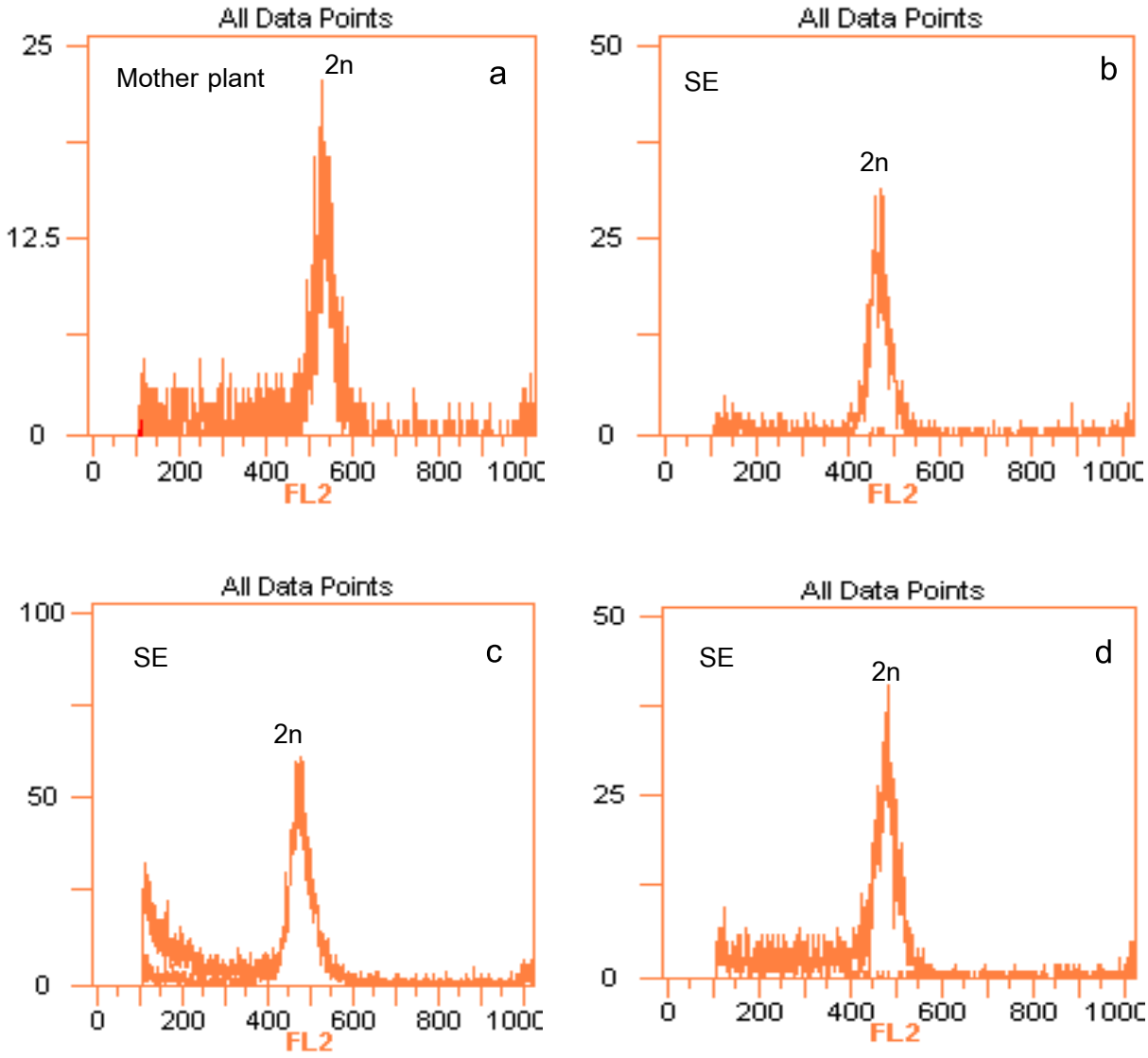

Figure 12 Determination of DNA content by flow cytometry. All sample showed the same peak as mother plant (a) at the specific chanel 450-500. (b-c) somatic embryos sample derived from anther culture on MS medium with 0.06 mg/l NAA, 0.03 mg/l BA, 3% sucrose and 0.75% agar for 8 weeks.

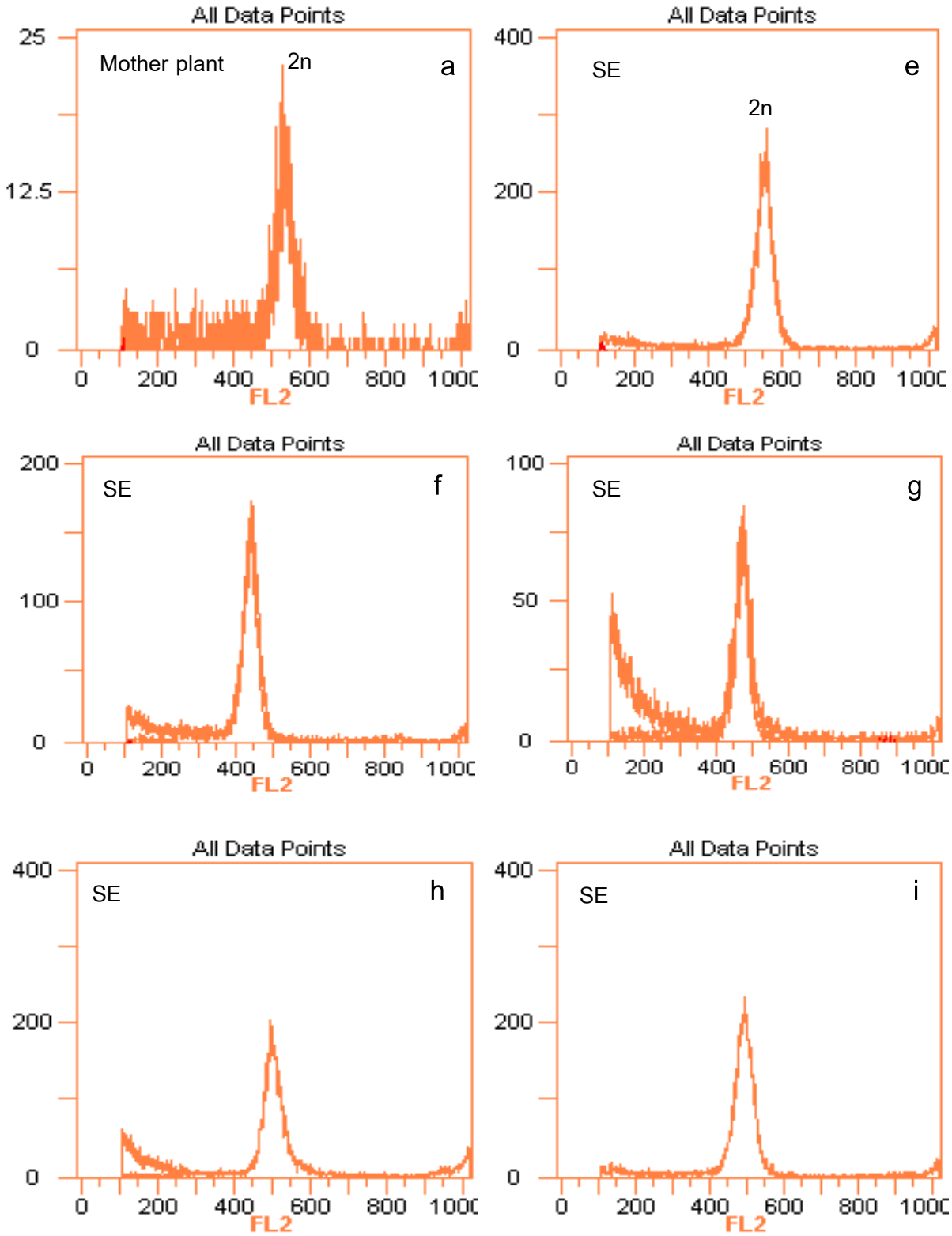

Figure 12 (continued) Determination of DNA content by flow cytometry. All sample showed the same peak as mother plant (a) at the specific chanel 450-500. (e-i) somatic embryos sample derived from anther culture on MS medium with 0.06 mg/l NAA, 0.03 mg/l BA, 3% sucrose and 0.75% agar for 8 weeks.

CHAPTER IV

Experiment II

Assessment of Somaclonal Variation of *In Vitro* Plantlets by RAPD and SSR markers

Introduction

Molecular markers have served as useful aids in understanding the genetics of *H. brasiliensis* in the recent year. They can play an important role in assisting *Hevea* clonal identification. For the last two decades, a large number of molecular markers and techniques have been applied in *Hevea* breeding (Venkatachalam *et al*., 2007). Several methods of molecular markers have been used with *H. brasiliensis.* For example, Besse *et al*. (1994) studied the genetic diversity of 92 Amazonian and 73 Wickham clones using RFLP analysis. Low *et al.* (1996) also used RFLPs for identification of progenies with two common parents. Varghese *et al.* (1997) evaluated 24 cultivated *Hevea* clones to estimate genetic distances. Subsequently, Venkatachalam *et al*. (2002) described the genetic relationships of 37 *Hevea* clones using RAPD markers. Lekawipat *et al*. (2003) evaluated the genetic relatedness of wild and cultivated *Hevea* accessions with SSCP. Identification of dwarf genome was performed by RAPD marker (Venkatachalam *et al*., 2004). Nakkanong *et al.* (2008) analyzed 53 early introduced clones of rubber tree collected from different areas in southern Thailand. The assessment was performed using RAPD and microsatellite markers. Their results showed that eight primers, OPB-17, OPN-16, OPR-02, OPR-11, OPZ-04, OPAD-01, OPAD-10 and OPAD-12, were chosen for genetic variation analysis in 87 individual plants. Four microsatellite primer pairs, *hmac*4, *hmct*1, *hmct*5 and *hmac*5, produced a total of 44 amplified fragments with an average of 14.67 fragments per primer, of which 37 (84.09%) were polymorphic while *hmac5* produced only monomorphic fragments. However, there are no molecular markers used for investigation of genetic instability in micropropagation of rubber tree. Therefore, in this experiment, RAPD and SSRs techniques were used to confirm the uniformity before cultivation in the field and further using for commercial screening abnormality of somaclones obtained from tissue culture technique of rubber tree. The profiles of DNA obtained from both RAPDs and SSRs can be determined

by PCR; the first technique was amplified with 10-mer primers and the second one with a pair of specific primers designed on microsatellite flanking regions. These kinds of DNA markers offer the advantage of being simpler to use, less expensive, and less time-consuming than other markers (Jin *et al*., 2008).

Materials and methods

Plant materials

In vitro plantlets derived from microcutting technique of one-month-old seedlings of early introduced clones (Figure 13) were used in this experiment. The early introduced rubber clones were recognized by their big trunk indicating an age of more than 50 years and their random located outside established rubber plantation areas (Nakkanong *et al.,* 2008). The cultures were maintained at 28±0.5^{o} C under fluorescent bulbs at 12.5 µmol/m^{2}/s for a 14 hour photoperiod. *In vitro* young leaves were used for DNA isolation.

Figure 13 *In vitro* rubber tree plantlets on root induction medium (RIM; MS medium supplemented with 5 mg/l IBA, 1 mg/l silver nitrate, 3% sucrose and 0.05% activated charcoal) for 6 weeks.

DNA extraction

Total genomic DNA was extracted from *in vitro* young leaves derived from microcutting method by the cetyltrimethyl ammonium bromide (CTAB) method of Doyle and Doyle (1990) with some modification. In brief, 100 mg of *in vitro* young leaf were ground to fine powder in approximately 500 µl of CTAB buffer. The plant extract mixtures were transferred to microcentrifuge tube and incubated at 60 °C in water bath for 45 minutes. After incubation, the extracted mixtures were centrifuged at 10,000 rpm for 15 minutes and transferred the supernatant to a new clean microcentrifuge tube. Each tube was added with 500 µl of chloroform and mixed the solution by inversing the tube. After mixing well, the tube was centrifuged at 12,000 rpm for 10 minutes and transferred the upper aqueous phase only (contains the DNA) to a clean microcentrifuge tube. The solution was added with 750 µl Isopropanol and inverted the tube slowly for several times to precipitate the DNA. After precipitation, DNA pellet was washed with 70% ethanol for two times and dried under room temperature. The DNA pellet was dissolved in TE buffer [20 mM Tris-HCl (pH 8.0) and 0.1M EDTA (pH 8.0)], electrophoresed on 0.75% agarose gel, exposed to UV light and photographed. The amount of DNA was qualitatively measured by comparison with known quantity of λ DNA.

RAPD analysis

RAPD analysis was performed according to the methodology of Nakkanong *et al.* (2008). Each amplification mixture of 25 µl contained 25 mM $MgCl_2$, 10x *Taq* buffer, 100 µM of each dNTP, 0.3 mM of primer (OPB-17, OPAN-16, OPR-02, OPR-11, OPZ-4, OPAD-01, OPAD-10 and OPAD-12), 1.5 units of *Taq* polymerase and 60 ng of template DNA. The thermal profile for RAPD-PCR was started at 94 °C for 2 minutes, followed by 41 cycles of 94 °C for 30 seconds 37 °C for 1 minute 72 °C for 2 minutes and finally 72 °C for 5 minutes. Amplification products were then separated by electrophoresis in 1.5% (w/v)

agarose gels in 0.5X TBE buffer at constant 100 V. The gels were stained with ethidium bromide for 15 minutes, immersed in distilled water for 5 minutes and viewed under ultraviolet light with gel documentation. The amplification products of DNA were compared among the samples.

SSR analysis

Three SSR primer pairs (*hmac4 hmct1* and *hmct5*) were used for PCR amplification of DNA following a protocol described by Thawaro and Te-chato (2008). Each amplification mixture of 10 µl contained 2.5 mM MgCl2, 10x *Taq* buffer, 100 µM of each dNTP, 0.3 mM of primer, 1.5 units of *Taq* polymerase and 20 ng of template DNA. PCR amplifications were carried out on a thermocycler (TC-XP-G, Japan) using the following program: denaturation at 95°C for 1 min; 35 cycles of 94°C for 30 s, 52°C for 60 s, and 72°C for 120 s; and a final elongation step at 72°C for 8 min. An equal volume of loading buffer (98% formamide, 0.025% bromophenol blue, 0.05% xylene cyanol) was added to the amplified products, following denaturation at 94°C for 5 min. The products were separated on 6% (w/v) denaturing polyacrylamide gels. DNA bands were visualized with silver nitrate.

Result and Discussion

In plant propagation, the most crucial aspect is to retain genetic integrity with respect to the mother plants (MP); however, it is known that *in vitro* culture techniques could induce genetic variability, namely somaclonal variation (Jin *et al*., 2008). While this is considered as a problem in commercial micropropagation, since it can negatively affect the productions and the uniformity and reduce the performance of elite genotypes (Palombi *et al*., 2007). Genetic changes in associated with tissue culture process may imply an advantage as a source of variability for new forms, or whether this variation implies a disadvantage for the propagation of a specific cultivar or transformed genotype, it is

important to achieve a rapid and easy method to assess the genetic instability of the propagated material (Minano *et al*., 2009). Numerous studies on somaclonal variation analysis have been developed using molecular techniques such as RAPD, SSR, AFLP and RFLP. The joint use of various types of primers is an excellent way of identifying genotypes (Meszaros *et al*., 2007).

In the present works, from eight RAPD-primers tested (OPB-17, OPAN-16, OPR-02, OPR-11, OPZ-4, OPAD-01, OPAD-10 and OPAD-12) but only five primers (OPAD-01, OPAD-10, OPAD-12, OPAB-17 and OPR-02) could amplify and provided monomorphic patterns of DNA among *in vitro* rubber plantlets. The number of bands for each primer varied from 3 to 6 with an average of 6.40 fragments per primer. The size of amplified products ranged from 100 to 1517 base pair in size. A total of 21 RAPD fragments were scored from the five random primers. An example of RAPD amplification was shown in Figure 14-18. This result is consistent with similar observations of SSR analysis. Three primer pairs (*hmac4 hmct1* and *hmct5*) could amplify and also provided monomorphic patterns of DNA. The number of bands for each primer varied from 4 to 5 with an average of 13.33 fragments per primer. The size of amplified products ranged from 100 to 650 base pair in size. A total of 13 SSRs fragments were scored from the three primer pairs (Figure 19-21). The results from the above two techniques revealed that there was no somaclonal variation occurred among *in vitro* rubber plantlets derived from our propagation system. The primers used in this study showed polymorphism among early introduced clones of rubber tree (*Hevea brasiliensis*) collected from different areas in southern Thailand (Nakkanong *et al.,* 2008). However, these primers provided monomorphic DNA patterns in this present study. Thus, *in vitro* rubber plantlets obtained from this propagation were uniformity. Previous report by Hua *et al*. (2010) indicated the abnormal embryos after *in vitro* anther culture of rubber tree (CATAS 7-33-97 and CATAS 88-13 clones).

However, these embryos obtained from this study could not develop into plantlets. Accordingly, they did not assess by molecular markers. In this study, somaclonal variation was not observed. *In vitro* rubber plantlets derived from microcutting in this present study provided the new shoots from shoot tips or nodal culture directly without passing callus formation. Thus, the frequency of somaclonal variation was lower than callus or somatic embryo-derived plantlets. Somaclonal variation is manifested as cytological abnormalities, frequent qualitative and quantitative phenotypic mutation, sequence change, and gene activation and silencing. Many factors have the relationship with the frequency of somaclonal variation (Kaeppler *et al*. 2000). Among them, the type of culture plays an important role in the genetic stability of *in vitro* cultures (Jin *et al*., 2008). Many researchers reported that BA was the most toxic cytokinin based on the abnormality index and it can be produced somaclonal variation in banana (Bairu *et al*., 2008). The role of cytokinins in embryo induction and development appears extremely complex (Siragusa *et al*., 2007). However, in our culture systems, high concentration of cytokinin (5 mg/l BA) does not have an effect on somaclonal variation but long-term multiplication may lead to somaclonal or epigenetic variations in micropropagated plants.

This is the first report on the use of molecular markers to assess somaclonal variation in rubber micropropagation. The uniformity of micropropagated plantlets could be distinguished using five RAPD or three microsatellite primer pairs. Both of these markers were suitable for the assessment of somaclonal variation. RAPD primers randomly scan the whole genome detecting DNA mutations. This analysis requires only a small amount of DNA and permits to analyze quickly and economically many samples (Siragusa *et a*l., 2007). SSR primers also provided a large quantity of polymorphic information. It detects genetic mutations at hypervariable sites, such as DNA repetitive regions, using highly specific 18-bp long primers. For this reason, this technique guarantees higher reliability and repeatability

than RAPD technique (Bornet and Branchard 2001). However, the joint use of various types of markers is an excellent way of identifying genetic variations generated by *in vitro* culture technique (Meszaros *et al*., 2007).

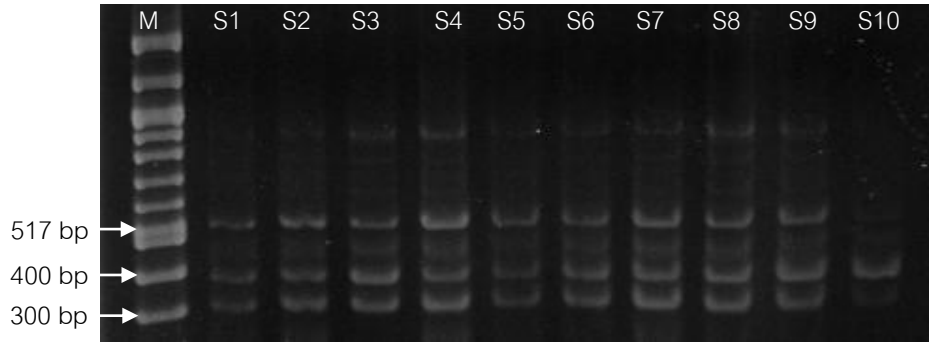

Figure 14 RAPD patterns of micropropagated plantlets amplified by primer OPAD-10. Lane M = 100 bp ladder, Lane S1-S10 = DNA of *in vitro* young leaves samples.

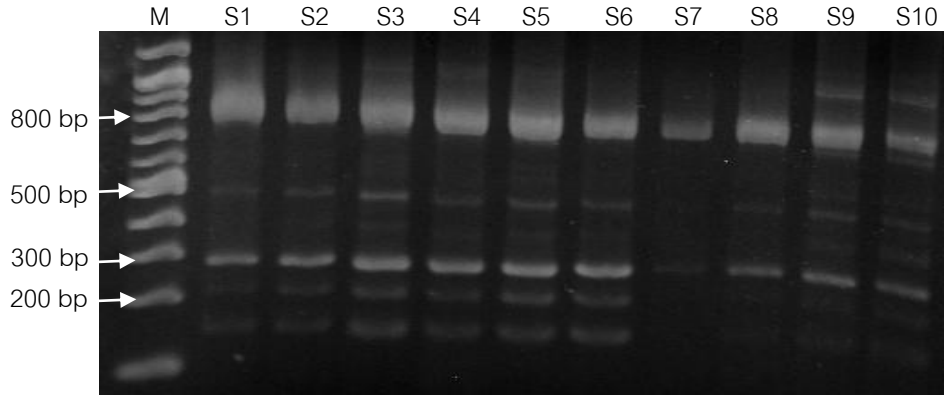

Figure 15 RAPD patterns of micropropagated plantlets amplified by primer OPAD-12. Lane M = 100 bp ladder, Lane S1-S10 = DNA of *in vitro* young leaves samples.

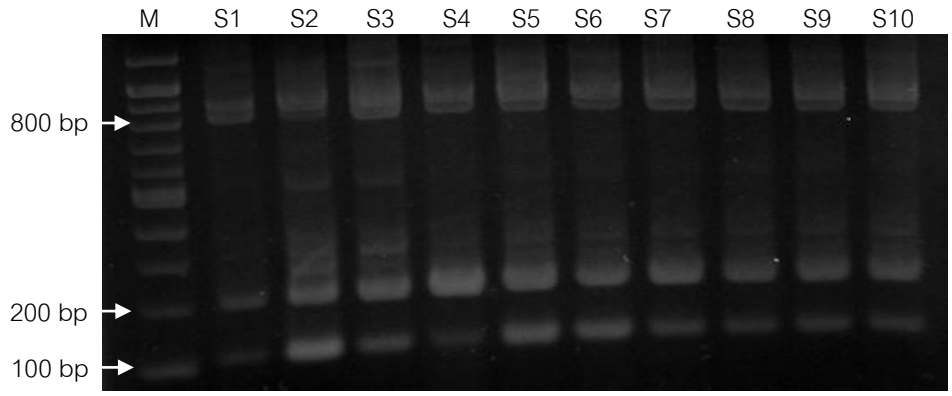

Figure 16 RAPD patterns of micropropagated plantlets amplified by primer OPAB-17. Lane M = 100 bp ladder, Lane S1-S10 = DNA of *in vitro* young leaves samples.

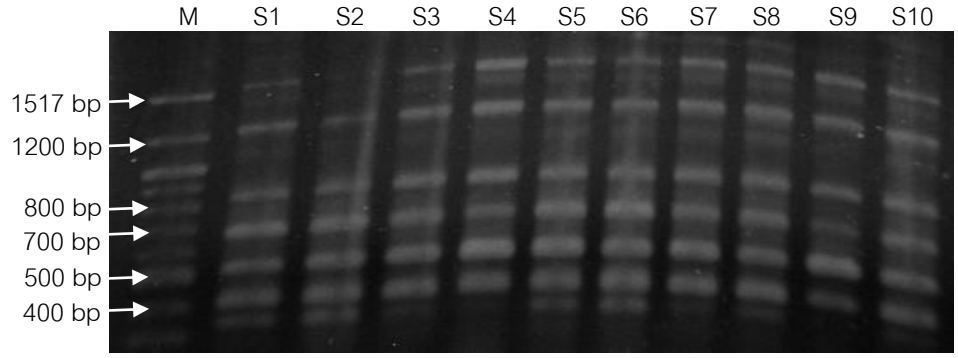

Figure 17 RAPD patterns of micropropagated plantlets amplified by primer OPAD-01. Lane M = 100 bp ladder, Lane S1-S10 = DNA of *in vitro* young leaves samples.

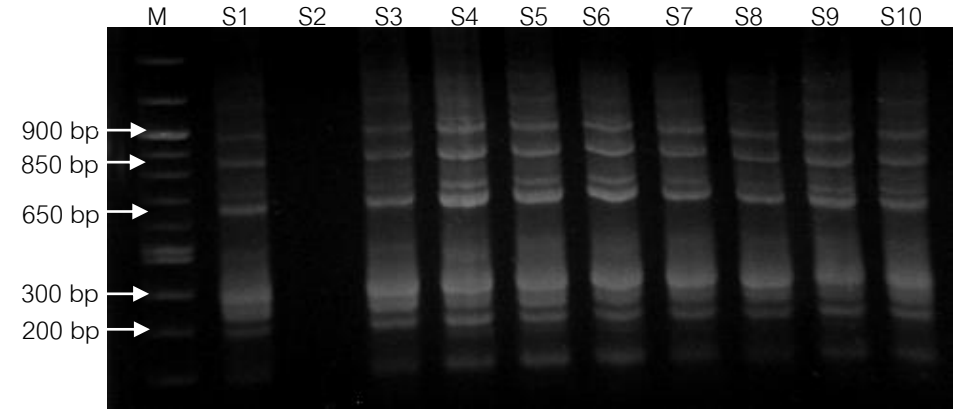

Figure 18 RAPD patterns of micropropagated plantlets amplified by primer OPR-02. Lane M = 100 bp ladder, Lane S1-S10 = DNA of *in vitro* young leaves samples.

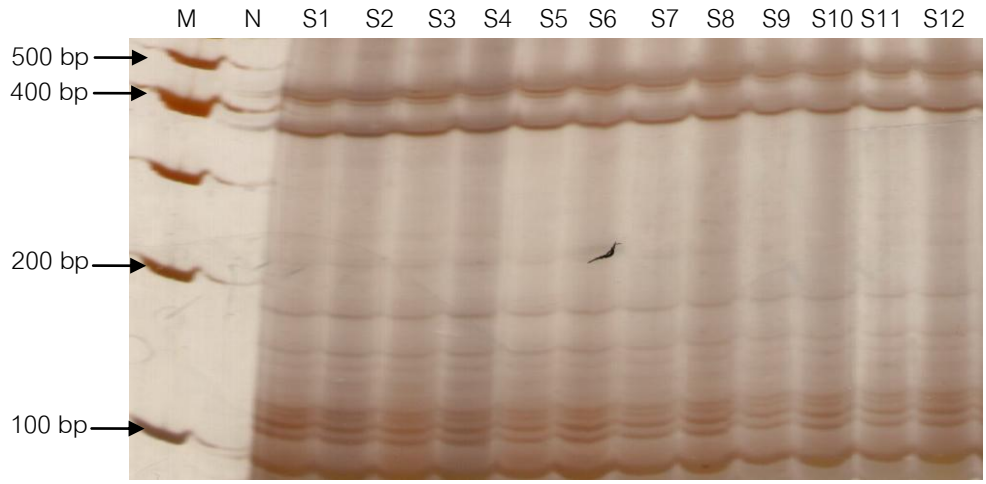

Figure 19 SSRs patterns of micropropagated plantlets amplified by primer *hmac4*. Lane M = 100 bp ladder, N = Negative control, Lane S1-S12 = DNA of *in vitro* young leaves samples.

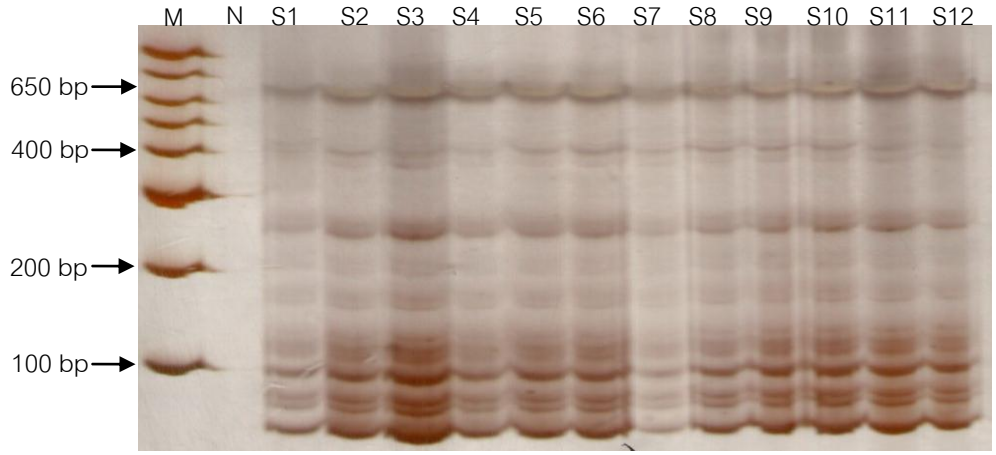

Figure 20 SSRs patterns of micropropagated plantlets amplified by primer *hmact1*. Lane M = 100 bp ladder, N = Negative control, Lane S1-S12 = DNA of *in vitro* young leaves samples.

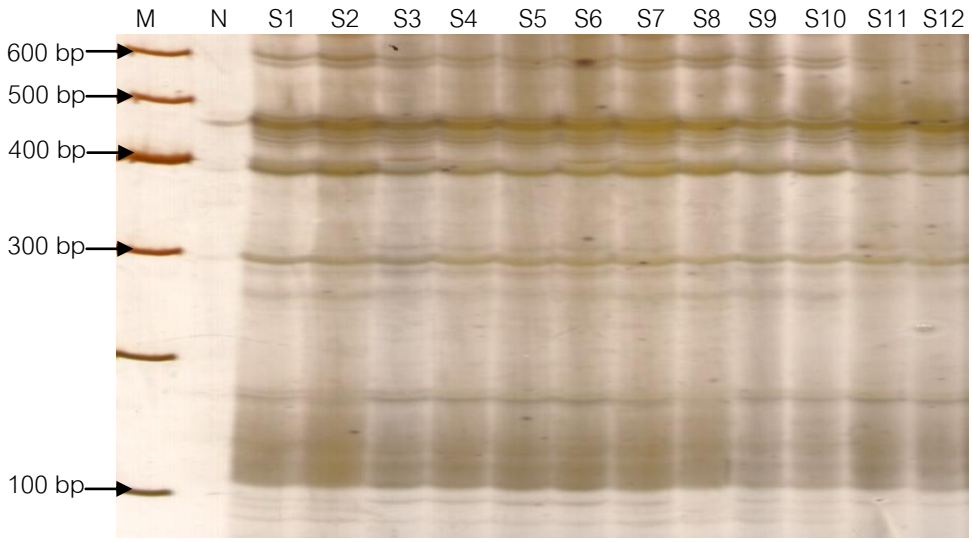

Figure 21 SSRs patterns of micropropagated plantlets amplified by primer *hmact5*. Lane M = 100 bp ladder, N = Negative control, Lane S1-S12 = DNA of *in vitro* young leaves samples.

CHAPTER V

Concluding Remarks

Concluding remarks

Shoot tips or cotyledonary nodes from one-month old *in vitro* seedling of rubber tree cultured on shoot induction medium (SIM; MS medium containing 5 mg/l BA, 0.5% activated charcoal, 3%sucrose and 0.75% agar) supplemented with $AgNO_3$ gave the better results in multiple shoot formation than the medium containing peptone. Multiple shoot were produced at 100% with a mean number of 5.60 ± 3.36 shoots per explant on SIM supplemented with 1 mg/l $AgNO_3$. The shoots had dark green leaves and grew vigorously. The shoots obtained from the medium without supplementation with $AgNO_3$ were pale green, and senescence of leaves was found after culture for 4 weeks. One-hundred percent root induction was obtained from the shoot explant cultured on root induction medium (RIM) which was MS medium supplemented with 5 mg/l IBA, 5 mg/l NAA, 0.05% activated charcoal, 1% $AgNO_3$, 3% sucrose and 0.75% agar.

For somatic embryogenesis, embryogenic callus was induced from immature anther on callus induction medium (CIM; MS medium containing 1 mg/l 2, 4-, 1 mg/l KN, 1 mg/l NAA and 3% sucrose). Optimum frequency of somatic embryogenesis at 64% and a number of somatic embryos (SEs) per explant at 5.13 SEs was achieved on MS medium supplemented with 0.06 mg/l NAA, 0.03 mg/l BA, 3% sucrose and 0.75% agar. These SEs from the medium showed the same ploidy level as mother plants after evaluation by flow cytometry. Unfortunately, these SEs could not develop into plantlet.

Assessment of somaclonal variation was carried out to detect uniformity of *in vitro* rubber tree plantlet derived from microcutting technique by RAPD and SSR markers. Plantlets obtained by this procedure had the same profiles of DNA patterns as revealed by 5 primers (OPAD-01, OPAD-10, OPAD-12, OPB-17 and OPR-02) of RAPD and 3 primers

(*hmac4*, *hmct1* and *hmct5*) of SSR marker. It was clear that somaclones obtained from our protocol was uniform and successfully used in micropropagation purpose.

Future prospect

In vitro techniques have great potential for propagation, collecting and conservation of genetic resources of rubber tree. An efficient plant regeneration system through microcutting technique of rubber tree established in this study opens a wide view to apply advanced biotechnology for rubber tree improvement. In case of somatic embryogenesis, its improvement can be carried out by the using of embryogenic callus or somatic embryos as initial explants for rubber tree breeding program as follows:

Mutation induction

Callus, somatic embryo and single shoot will be suitable explants for induction of mutation by chemical or physical mutagen. These treated explants will be regenerated into plantlets and examined by morphological, cytological, biochemical and molecular techniques to confirm their genetic instability.

Genetic transformation

Callus, somatic embryo and single shoot of rubber tree will be transformed by some important genes using *Agrobacterium*-mediated transformation or particle bombardment. Transformants will be detected by histochemical, resistance to some chemical according to the nature of reporter genes in the plasmid or analysis by molecular markers. Molecular mechanisms involved in rubber production are an essential avenue for the genetic improvement of rubber tree by conventional methods or by trangenesis.

Generally, genetic improvement in rubber tree by conventional breeding is very slow and time-consuming, as in many other perennial crops. Mutation induction and

genetic engineering are certainly a more promising method for a high efficiency of crop improvement when specific genetic changes need to be made in a short time period.

REFERENCES

Ayana, A., Bekele, E. and Bryngelsson, T. 2002. Genetic variation in wild sorghum (*Sorghum bicolor*) germplasm from Ethiopia assessed by random amplified polymorphic DNA (RAPD). Hereditas 132: 249-254.

Bairu, M., Stirk, W. A., Dolezal, K. and Staden, J. 2008. The role of topolins in micropropagation and somaclonal variation of banana cultivars 'Williams' and 'Grand Naine' (*Musa* spp. AAA). Plant Cell, Tissue and Organ Culture 95: 373-379.

Barakat, M. N. and El-Sammak, H. 2011. *In vitro* mutagenesis, plant regeneration and characterization of mutants via RAPD analysis in Baby's breath *Gypsophila paniculata* L. Australian Journal of Crop Science 5: 214-222.

Besse, P., Seguin, M., Lebrun, P., Chevallier, M.H., Nicolas, D. and Lanaud, C. 1994. Genetic diversity among wild and cultivated populations of *Hevea brasiliensis* assessed by nuclear RFLP analysis. Theoretical and Applied Genetics 88: 199-207.

Bornet, B. and Branchard, M. 2001. Non-anchored inter simple sequence repeat (ISSR) markers: reproducible and specific tools for genome fingerprinting. Plant Molecular Biology Reporter 19: 209–215.

Bouychou, J. G. 1953. La culture *in vitro* des tissue *Hevea.* Proceedings Rubber Conference Bogor, Archives of Rubber Cultivation 30: 50-53.

Cesar, A.H., Lucia, A.K., Rafael, A.I. and Mario, L.A. 2006. Analysis of genetic variation in clones of rubber (*Hevea brasiliensis*) from Asian, South and Central American origin using RAPD markers. Revista Colombiana de Biotechnologia 8: 29-34.

Chen, J.T. and Chang, W.C. 2002. Effects of tissue culture conditions and explant characteristics on direct somatic embryogenesis in *Oncidium* 'Gower Ramsey'. Plant Cell, Tissue and Organ Culture 69: 41-44.

Cogbill, S., Faulcon, T., Jones, G., McDaniel, M., Harmon, G., Blackmon, R. and Young, M. 2010. Adventitious shoot regeneration from cotyledonary explants of rapid-cycling fast plants of *Brassica rapa* L. Plant Cell, Tissue and Organ Culture 101: 127-133.

Doyle, J.J. and Doyle, J.L. 1990. Isolation of plant DNA from fresh tissue. Focus 12: 13-15.

Dickson, A. I., Okere, A., Elizabeth, J., Mary, O., Olatunde, F. and Abiodun, S. 2011. *In-vitro* culture of *Hevea brasiliensis* (rubber tree) embryo. Journal of Plant Breeding and Crop Science 3: 185-189.

Ehsanpour, A. A., Madini, S. and Hoseini, M. 2007. Detection of somaclonal variation in potato callus induced by UV-C radiation using RAPD-PCR. General and Applied Plant Physiology 33: 3-11.

Feng, J.C., Yu, X.M., Shang, X.L., Li, J.D. and Wu, Y.X. 2010. Factors influencing efficiency of shoot regeneration in *Ziziphus jujuba* Mill. 'Huizao'. Plant Cell, Tissue and Organ Culture 101: 111-117.

Fiuk, A. and Rybczynski, J. J. 2008. Genotype and plant growth regulator dependent response of somatic embryogenesis from *Gentiana* spp. leaf explants. In Vitro Cellular and Developmental Biology-Plant 44:90–99.

Gondo, T., Tsuruta, S., Akashi, R., Kawamura, O. and Hoffmann, F. 2005. Green, herbicide-resistant plants by particle inflow gun-mediated gene transfer to diploid bahiagrass (*Paspalum notatum*). Journal of Plant Physiology 162: 1367-1375.

Gu, X. F., Yang, A. F., Meng, H. and Zhong, J. R. 2005. *In vitro* induction of tetraploid plants from diploid *Zizyphus jujube* Mill. cv. Zhanhua. Plant Cell Reports 24: 671–676.

Hua, Y. W., Huang, T. D. and Huang, H. S. 2010. Micropropagation of self-rooting juvenile clones by secondary somatic embrogenesis in *Hevea brasiliensis*. Plant Breeding 129: 202-207.

Ishigaki, G., Gondo, T., Suenaka, K. and Akashi, R. 2009. Induction of tetraploid ruzigrass (*Brachiaria ruziziensis*) plants by colchicine treatment of in vitro multiple-shoot clumps and seedlings. Grassland Science 55: 164-170.

Jayashree, P. K., Asokan, M. P., Sobha, S., Ammal, L. S., Rekha, K., Kala, R. G., Jayasree, R. and Thulaseedharan, A. 1999. Somatic embryogenesis and plant regeneration from immature anthers of *Hevea brasiliensis* Muell. Arg. Current Science 76: 1242-1245.

Jin, S., Mushke, R., Zhu, H., Tu, L., Lin, Z., Zhang, Y. and Zhang, X. 2008. Detection of somaclonal variation of cotton (*Gossypium hirsutum*) using cytogenetics, flow cytometry and molecular markers. Plant Cell Reports 27: 1303-1316.

Kaeppler, S. M., Kaeppler, H. F. and Rhee, Y. 2000. Epigenetic aspects of somaclonal variation in plants. Plant Molecular Biology 43:179–88

Karacsonyi, D., Chiru, N. and Nistor, A. 2011. Microsatellite analysis of somaclonal variation in potato (*Solanum tuberosum ssp. tuberosum*) plantlets regenerated from callus. Romanian Biotechnological Letters 16: 81-83.

Khonglao, K. 2006. Study of root growth and root profile in ten rubber clones (*Hevea brasiliensis*). Special Problem Report, Department of Plant Science, Faculty of Natural Resources, Prince of Songkla University, Songkhla, Thailand.

Kouassi, K. M., Koffi, K. E., Gnagne, Y. M., N'nan, O., Coulibaly, Y. and Sangare, A. 2008. Production of *Hevea brasiliensis* embryos from *in vitro* culture of unpolinated ovules. Biotechnology 7: 793-797.

Lardet, L., Martin, F., Dessailly, F., Carron, M.P. and Montoro, P. 2007. Effect of exogenous calcium on post-thaw growth recovery and subsequent plant regeneration of cryopreserved embryogenic calli of *Hevea brasiliensis* Muell. Arg. Plant Cell Reports 26: 559-569.

Lekawipat, N., Teerawatanasuk, K., Vanavichit, A., Toojinda, T. and Tragoonrung, S. 2003. Genetic diversity analysis of wild germplasm and cultivated clones of *Hevea brasiliensis* Muell. Arg. by using microsatellite marker. Journal of Rubber Research 6: 36 - 47.

Lema-Ruminska, J. 2011. Flow cytometric analysis of somatic embryos, shoots, and calli of the cactus *Copiapoa tenuissima* Ritt. *forma monstruosa*. Plant Cell, Tissue and Organ Culture 106: 531-535.

Low, F. C., Atan, S., Jaafar, H. and Tan, H. 1996. Recent advances in the development of molecular markers for *Hevea* studies. Journal of Natural Rubber Research 11: 32-44.

Luo, H., Coppenolle, B.V., Seguin, M. and Boutry, M. 1995. Mitochondrial DNA polymorphism and phylogenetic relationships in *Hevea brasiliensis*. Molecular Breeding 1: 51-63.

Mahmood, T., Naza, N., Abbasi, B. H., Khan, M. A., Ahmad, M. and Zafar, M. 2010. Detection of somaclonal variations using RAPD fingerprinting in *Silybum marianum* (L.). Journal of Medicinal Plants Research 4: 1822-1824.

Mendanha, A. B. L., Torres, A. de A. and Freire, A. de Barros.1998. Micropropagation of rubber trees (*Hevea brasiliensis* Muell. Arg.). Genetic and Molecular Biology 21: 14-15.

Meszaros, K., Karsai, I., Kuti, C., Banyai, J., Lang, L. and Bedo, Z. 2007. Efficiency of different marker systems for genotype fingerprinting and for genetic diversity studies in barley (*Hordeum vulgare* L.). South African Journal of Botany 73: 43-48.

Minano, H. S., Gonzalez-Benito, M. E. and Martin, C. 2009. Molecular characterization and analysis of somaclonal variation in chrysanthemum cultivars using RAPD markers. Scientia Horticulturae 122: 238-243.

Montoro, P., Rattana, W., Pujade-Renaud, V., Michaux-Ferriere, N., Monkolsook, Y., Kanthapura, R. and Adunsadthapong, S. 2003. Production of *Hevea brasiliensis* transgenic embryogenic callus lines by *Agrobacterium tumefaciens*: roles of calcium. Plant Cell Reports 21: 1095-102.

Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiologia Plantarum 15: 473-497.

Nakkanong, K., Nualsri, C. and Sdoodee, S. 2008. Analysis of genetic diversity in early introduced clones of rubber tree (*Hevea brasiliensis*) using RAPD and microsatellite markers. Songklanakarin Journal of Science and Technology 30: 553-560.

Nayanakantha, N. M. C. and Seneviratne, P. 2007. Tissue culture of rubber: past, present and future prospects. Ceylon Journal of Science (Biological Science) 36: 116-125.

Nhut, D.T., Thi, N.N., Khiet, B.L.T. and Luan, V.Q. 2008. Peptone stimulates in vitro shoot and root regeneration of avocado (*Persea americana* Mill.). Scientia Horticulturae 115: 124-128.

Nunez, R. 2001. DNA measurement and cell cycle analysis by flow cytometry. Current Issues in Molecular Biology 3: 67-70.

Palombi, M. A. and Damiano, C. 2002. Comparison between RAPD and SSR molecular markers in detecting genetic variation in kiwifruit (*Actinidia deliciosa* A. Chev). Plant Cell Reports 20: 1061-1066.

Palombi, M. A., Lombardo, B. and Caboni, E. 2007. In vitro regeneration of wild pear (*Pyrus pyraster* Burgsd) clones tolerant to Fe-chlorosis and somaclonal variation analysis by RAPD markers. Plant Cell Reports 26: 489-496.

Parimalan, R., Giridhar, P. and Ravishankar, G. A. 2010. Enhanced shoot organogenesis in *Bixa orellana* L. in the presence of putrescine and silver nitrate. Plant Cell, Tissue and Organ Culture 105: 285-290.

Pinto, D. L. P., Barros, B. de A., Viccini, L. F., Campos, J. M. de S., Silva, M. L. and Otoni, W. C. 2010. Ploidy stability of somatic embryogenesis-derived *Passiflora cincinnata* Mast. plants as assessed by flow cytometry. Plant Cell, Tissue and Organ Culture 103: 71-79.

Roy, C. B., Nazeer, M. A. and Saha, T. 2004. Identification of simple sequence repeats in rubber (*Hevea brasiliensis*).Current science 87: 807-811.

Saengruksawong, C., Soontorn K., Niwat, A. and Jitti P. 2012. Growths and carbon stocks of para rubber plantations on Phonpisai soil series in northeastern Thailand. Rubber Thai Journal 1:1-8.

Saha, T., Roy, C. B. and Nazeer, M. A. 2005. Microsatellite variability and its use in the characterization of cultivated clones of *Hevea brasiliensis*. Plant Breeding 124: 86 - 92.

Seeni, S. and Latha, P.G. 1992. Foliar regeneration of the endangered Red Vanda, *Renanthera imschootiana* Rolfe (Orchidaceae). Plant Cell, Tissue and Organ Culture 29: 167-172.

Sheidai, M., Aminpoor, H., Noormohammadi, Z. and Farahani, F. 2008. RAPD analysis of somaclonal variation in banana (*Musa acuminate* L.) cultivar Valery. Acta Biologica Szegediensis 52: 307-311.

Siragusa, M., Carra, A., Salvia, L., Puglia, A. M., Pasquale, F and Carimi, F. 2007. Genetic instability in calamondin (*Citrus madurensis* Lour.) plants derived from somatic embryogenesis induced by diphenylurea derivatives. Plant Cell Reports 26: 1289-1296.

Sushamakumari, S., Asokan, M.P., Anthony, P., Lowe, K.C., Power, J.B. and Davey, M.R. 2000. Plant regeneration from embryogenic cell suspension-derived protoplasts of rubber. Plant Cell, Tissue and Organ Culture 61: 81–85.

Te-chato, S. 1997. Tissue culture of mangosteen (*Garcinia mangostana* L.), pawa (*G. speciosa* Wall.) and somkhag (*G. atroviridis* Griff.). Songklanakarin Journal of Science and Technology 19: 147-155.

Te-chato, S. and Muangkaewngam, A. 1992. Tissue culture of rubber I: *In vitro* micropropagation of rubber. Songklanakarin Journal of Science and Technology 14: 123-132.

Te-chato, S. and Chartikul, M. 1993. Tissue culture of rubber: Certain factors affecting callus formation from integument seed. Songklanakarin Journal of Science and Technology 15: 227-233.

Te-chato, S., Niyagij, C. and Suranilpong, P. 2002. Callus formation from protoplasts derived from cell suspension culture of rubber tree (*Hevea brasiliensis* Muell. Arg.). Thai Journal of Agricultural Science 35: 165-173.

Thawaro, S. and Te-chato, S. 2009. Verification of legitimate tenera oil palm hybrids using SSR and propagation of hybrids by somatic embryogenesis. Songklanakarin Journal of Science and Technology 32: 1-8.

Tingey, S.V. and Tufo, J.P. 1993. Genetic analysis with random amplified polymorphic DNA markers. Plant Physiology 101: 439–452.

Varghese, Y. A., Knaak, C., Sethuraj, M. R. and Ecke, W. 1997. Evaluation of random amplified polymorphic DNA (RAPD) markers in *Hevea brasilinesis*. Plant Breeding 116: 47-52.

Venkatachalam, P., Jayasree, P. K., Sushmakumari, S., Jayashree, R., Sobha, S., Priya, P., Kala, R. G. and Thulaseedharan, A. 2007. Current perspectives on application of biotechnology to assist the genetic improvement of rubber tree (*Hevea brasiliensis* Muell. Arg.): An overview. Functional Plant Science and Biotechnology 1: 1-17.

Venkatachalam, P., Priya, P., Gireesh, T., Saraswathy Amma, C. K. and Thulaseedharan, A. 2006. Molecular cloning and sequencing of a polymorphic band from rubber tree [*Hevea brasiliensis* (Muell.) Arg.]: the nucleotide sequence revealed partial homology with proline-specific permease gene sequence. Current Science 90: 1510-1515.

Venkatachalam, P., Priya, P., Saraswathy Amma, C. K. and Thulaseedharan, A. 2004. Identification, cloning and sequence analysis of a dwarf genome-specific RAPD marker in rubber tree [*Hevea brasiliensis* (Muell.) Arg.]. Plant Cell Reports 23: 327-332.

Venkatachalam, P., Thomas, S., Priya, P., Thanseem, I., Gireesh, T., Saraswathyamma, C. K. and Thulaseedharan, A. 2002 Identification of DNA polymorphism with the cultivated clones of rubber tree (*Hevea brasiliensis* Muell. Arg.). Indian Journal of Natural Rubber Research 15: 72-181.

Vikrant and Rashid, A. 2002. Somatic embryogenesis from immature and mature embryos of a minor millet *Paspalum scrobiculatum* L. Plant Cell, Tissue and Organ Culture 69: 71-77.

Wang, Z., Wu, H., Zeng, X., Chen, C. and Li, Q. 1984. Embryogeny and origin of anther plantlet of *Hevea brasiliensis*. Chinese Journal of Tropical Crops 5: 9-13.

Williams, J., Pink, D.A.C. and Biddington, N.L. 1990. Effect of silver nitrate on long-term culture and regeneration of callus from *Brassica oleracea* var. *gemmifera*. Plant Cell, Tissue and Organ Culture 21: 61-66.

Yang, X. M., Cao, Z. Y., An, L. Z., Wang, Y. M. and Fang, X. W. 2006. *In vitro* tetraploid induction via colchicine treatment from diploid somatic embryos in grapevine (*Vitis vinifera* L.). Euphytica 152: 217–224.

Yang, X. M., An, L. Z., Xiong, Y. C., Zhang, J. P. L. I. Y. and Xu, S. J. 2008. Somatic embryogenesis from immature zygotic embryos and monitoring the genetic fidelity of regenerated plants in grapevine. Plant Biology 52:209–214.

Zhang, P., Phansiri, S. and Puonti, K.J. 2001. Improvements of cassava shoot organogenesis by the use of silver nitrate *in vitro*. Plant Cell, Tissue and Organ Culture 67: 47-54.

Zhou, Q. N., Jiang, Z. H., Huang, T. D., Li, W. G., Sun, A. H., Dai, X. M. and Li, Z. 2010. Plant regeneration via somatic embryogenesis from root explants of *Hevea brasiliensis*. African Journal of Biotechnology 9: 8168-8173.

APPENDICES

Appendix A

Table 1 Composition of nutrition of Murashige and Skoog (MS) medium

Components	mg/l
Macro elements	
NH_4NO_3	1,650.000
KNO_3	1,900.000
KH_2PO_4	170.000
$CaCl_2.2H_2O$	440.000
$MgSO_4.7H_2O$	370.000
Micro elements	
KI	0.830
H_3BO_3	6.200
$MnSO_4.H_2O$	16.900
$ZnSO_4.7H_2O$	10.600
$CuSO_4.5H_2O$	0.025
$Na_2MoO_4.2H_2O$	0.250
$CoCl_2.6H_2O$	0.025
$FeSO_4.7H_2O$	27.800
Na_2EDTA	37.300
Organic compounds	
Myo-inositol	100.000
Nicotinic acid	0.500
Pyridoxine HCl	0.500
Thaiamine HCl	0.100
Glycine	2.000
Sucrose	30,000.00
Agar	7,500.00
pH	5.7

Appendix B

Preparation of solution buffers and reagents for molecular markers

1. DNA isolation by the modified CTAB method

1.1 CTAB (Cetyltrimethyl ammonium bromide) buffer, 100 ml

- 10 ml of 1 M Tris HCl pH 8.0
- 8.12 g of $NaCl_2$
- 4 ml of 0.5 M Na_2EDTA (pH 8.0)
- 1 g of PVP-40
- 20 g of CTAB (cetyltrimethyl ammonium bromide)

Bring total volume to 100 ml with dH_2O. Add 2 g of CTAB and put in the water bath at 60°C for 15-20 minutes to dissolve the CTAB (Don't shake the solution – the detergent will bubble up too much). Sterilize by autoclaving. Prior to starting extraction, add 2% β-mercaptoethanol in the buffer. Once these have been added the shelf life of the buffer is only 2-3 days.

1.2 1.0 M Tris-HCl (pH 8.0)

- 121.1 g of Tris-HCl

Dissolve in about 700 ml of H_2O. Bring pH down to 8.0 by adding concentrated HCl.

1.3 TE buffer

- 500 µl of 1.0 M Tris-HCl (pH 7.5)
- 200 µl of 0.25M Na_2EDTA (pH 7.0)

Adjust the volume to 500 ml with distilled water and sterilize by autoclaving.

1.4 5X TAE buffer

- 121.1g of Tris Base
- 28.5 ml of Acetic acid

- 50.0 ml of 0.5M Na2EDTA (pH 8.0)

Adjust the volume to 500 ml with distilled water and sterilize by autoclaving. To make 1X TAE buffer before using.

1.5 5X TBE buffer

- 216 g of Tris Base
- 110 g of Boric acid
- 80 ml of 0.5M Na2EDTA (pH 8.0)

Adjust the volume to 4 L with distilled water and sterilize by autoclaving. To make 0.5X TBE buffer before using.

2. Solution for DNA staining by silver nitrate

2.1 Fixative and stop solution (10% acetic acid), 1,000 ml

- Glacial acetic acid 100 ml

Adjust volume to 1,000 ml with distilled water

2.2 0.2% Silver nitrate, 1,000 ml

- Silver nitrate 2.0 g

Adjust volume to 1,000 ml with distilled water

2.3 Develop solution, 1,000 ml

- Sodium carbonate 25 g

Adjust volume to 1,000 ml with distilled water, keep at 4 °C and add 500 µl of 40% formaldehyde and 40 µl of 50 mg / µl of sodium thiosulfate before using.

Appendix C

DNA sequences of primers

Table 1 RAPD primers

Primer	Sequences (5' → 3')	Pattern	Monomorphic fragment
OPB-17	AGGGAACGAG	monomorphic	3
OPAN-16	AAGCGACCTG	not clear	-
OPR-02	CACAGCTGCC	monomorphic	5
OPR-11	GTAGCCGTCT	not clear	-
OPZ-04	AGGCTGTGCT	not clear	-
OPAD-01	CAAAGGGCGG	monomorphic	6
OPAD-10	AAGAGGCCA	monomorphic	3
OPAD-12	AAGAGGGCGT	monomorphic	4
Total band scored			21

Table 2 SSR primers

Primer	Sequences (5' →3')	Pattern	Monomorphic fragment
hmac4	GTTTTCCTCCGCAGACTCAG(L)	monomorphic	4
	ATCCACCAAATAAGGCATGA(R)		
hmct1	TCGGTTGGTTTACCATGACA(L)	monomorphic	4
	ACATCACATGAGTGTATCTGATCTC(R)		
hmct5	ATGTATGTGTGCGCAGGAAG(L)	monomorphic	5
	CTGTAGTCATGGCAGCAGGT(R)		
Total band scored			13

Appendix D

Publications and Proceedings

Sirisom, Y. and Te-chato, S. 2012. The effect of peptone and silver nitrate on *in vitro* shoot formation in *Hevea brasiliensis* Muell Arg. International Journal of Agricultural Technology 8: 1509-1516.

Sirisom, Y. and Te-chato, S. 2013. Evaluation of anther-derived somatic embryos in *Hevea brasiliensis* Muell Arg. by flow cytometry. International Journal of Agricultural Technology 9: 713-720.

Sirisom, Y. and Te-chato, S. 2013. Assessment of somaclonal variation of rubber tree derived from microcutting by RAPD marker. The 12th National Toward AEC Under Climate Changes. Bangkok International Trade & Exhibition Centre, Bangkok, Thailand. 9-12 May 2013. pp. 24. (Oral presentation).

Sirisom, Y. and Te-chato, S. 2013. *In vitro* nodal culture of rubber tree and assessment of somaclonal variation by SSR marker. The 1st From Plant Science to AEC. Department of Plant Science, Faculty of Natural Resources, Prince of Songkla University, Hat Yai, Thailand. 13-14 August 2013. pp. 3. (Oral presentation).

PAPER 1

The effect of peptone and silver nitrate on *in vitro* shoot formation in *Hevea brasiliensis* Muell Arg.

PAPER 2

Evaluation of anther-derived somatic embryos in *Hevea brasiliensis* Muell Arg. by flow cytometry

PAPER 3

Assessment of somaclonal variation of rubber tree derived from microcutting by RAPD marker

PAPER 4

In vitro nodal culture of rubber tree and assessment of somaclonal variation by SSR marker

VITAE

Name Miss Yupaporn Sirisom

Student ID 5310630021

Education Attainment:

Degree	Name of Institution	Year of Graduation
Bachelor of Science (Biology)	Thaksin University	2004
Master (Plant Science)	Prince of Songkla University	2008

Scholarship Award during Enrolment:

1. The Scholarships for Ph.D. Program from the National Research University Project of Thailand's Office of the Higher Education Commission, Prince of Songkla University.
2. The Thesis Research Fund through the Graduate School, Prince of Songkla University.
3. The Partial Research Fund through the Center of Excellence in Agricultural and Natural Resources Biotechnology, Faculty of Natural Resources, Prince of Songkla University.

Work – Position and Address

Work-Position	Address	Year
Research Assistant	Department of Food Science, Faculty of Agro-Industry, Prince of Songkla University, Hat-Yai, Songkhla, Thailand	2008-2009
Research Assistant	Crop Biotechnology Laboratory, Department of Plant Science, Faculty of Natural Resources, Prince of Songkla University, Hat-Yai, Songkhla, Thailand	2009-2010