Propagation of Rubber Tree Resistant to White Root Disease through Somatic Embryogenesis from Thin Cell Layer and Floral Explants and Assessment of Somaclonal Variation by RAPD and SSR Markers

Kanjanee Tongtape

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

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ชื่อวิทยานิพนธ์ การขยายพันธุ์ยางพาราที่ทนทานต่อโรครากขาว โดยผ่านกระบวนการ Somatic Embryogenesis จาก Thin Cell Layer และชิ้นส่วนดอก และตรวจสอบความแปรปรวนทางพันธุกรรมด้วยเครื่องหมายโมเลกุล RAPD และ SSR

ผู้เขียน นางสาวกาญจนี ทองเทพ

สาขาวิชา พืชศาสตร์

ปีการศึกษา 2564

บทคัดย่อ

ยางพาราเป็นพืชที่สำคัญต่อเศรษฐกิจของประเทศไทย ในปัจจุบันการปลูกยางพาราได้ประสบปัญหาการแพร่ระบาด และการเข้าทำลายจากโรครากขาว ดังนั้นการใช้ต้นตอที่ทนทานต่อโรครากขาวจากยางพาราพันธุ์ดั้งเดิม เป็นการแก้ปัญหาดังกล่าวได้อย่างยั่งยืน จึงจำเป็นต้องเพิ่มปริมาณต้นตอยางที่ทนทานต่อโรคขาวให้เพียงพอต่อเกษตรกร งานวิจัยนี้จึงมีวัตถุประสงค์เพื่อศึกษาผลของสารควบคุมการเจริญเติบโต และชนิดของชิ้นส่วนพืชเพื่อเพิ่มปริมาณต้นตอยางพาราที่ทนทานต่อโรครากขาวในหลอดทดลอง เพื่อใช้ในการพัฒนาเป็นพืชต้นใหม่ในหลอดทดลอง โดยนำกิ่งยางอ่อนและดอกยางมาทำการฟอกฆ่าเชื้อด้วยโซเดียมไฮโปคลอไรท์ (NaOCl) ความเข้มข้น 0.525 เปอร์เซ็นต์ แล้วนำกิ่งอ่อนมาตัดเป็นชิ้นบางๆ ตามยาว (Longitudinally Thin Cell Layer; lTCL) กลีบดอก สับ 20 ครั้งต่อดอก และกลุ่มดอก สับ 60 ครั้งต่อ 3 ดอก วางเลี้ยงบนอาหาร Murashige and Skoog (MS) ร่วมกับ 6-benzyladenine (BA) และ2,4-Dichlorophenoxyacetic acid (2,4-D) ความเข้มข้นต่างๆ เป็นเวลา 4 สัปดาห์ พบว่า อาหารสูตรที่เติม BA เข้มข้น 2 มิลลิกรัมต่อลิตร ร่วมกับ 2, 4-D เข้มข้น 1.5 มิลลิกรัมต่อลิตร ให้อัตราการปลอดเชื้อ 100 เปอร์เซ็นต์ และอัตราการเกิดแคลลัส 100 เปอร์เซ็นต์ แคลลัสจากชิ้นส่วนของดอกมีลักษณะเกาะกันหลวมๆ (Friable Callus; FC) และเกาะกันแน่น (Compact Callus; CC) ในขณะที่แคลลัสจาก lTCL มีลักษณะเป็น CC เท่านั้น แคลลัสที่พัฒนาจากทุกชิ้นส่วนที่เพาะเลี้ยงมีสีเหลืองอมเขียว แคลลัสที่ชักนำจากกลุ่มดอกเพิ่มปริมาณได้ดีที่สุดให้น้ำหนักสดสูงสุด 392.05 มิลลิกรัม บนอาหารสูตรชักนำแคลลัสหลังจากเพาะเลี้ยงเป็นเวลา 4 สัปดาห์ แคลลัสดังกล่าวให้อัตราการเกิดโซมาติกเอ็มบริโอ (Somatic embryo; SE) สูงสุด 39.84 เปอร์เซ็นต์ จำนวน SE ระยะสร้างใบเลี้ยง (Cotyledonary embryo; CE) 3.25 เอ็มบริโอต่อหลอด หลังจากเพาะเลี้ยงเป็นเวลา 12 สัปดาห์ เมื่อนำ CE วางเลี้ยงบนอาหารสูตรเดิมที่เติม GA_3 ความเข้มข้นต่างๆ พบว่า GA_3 เข้มข้น 0.25 มิลลิกรัมต่อลิตร ให้อัตราการเกิดแกนต้นอ่อน 50 เปอร์เซ็นต์ และยอด 25 เปอร์เซ็นต์ หลังวางเลี้ยง 4 สัปดาห์ การตรวจสอบความแปรปรวนทางพันธุกรรม ของโซ

มาติกเอ็มบริโอโดยชักนำจากชิ้นส่วนที่แตกต่างกันเปรียบเทียบกับต้นแม่ พบว่า โซมาติกเอ็มบริโอที่ชักนำได้มีรูปแบบของดีเอ็นเอที่เหมือนกันจากการตรวจสอบด้วยเครื่องหมาย RAPD ใช้ไพรเมอร์ 2 ชนิด (OPAD-01 และ OPAD-10) และเครื่องหมาย SSR โดยใช้ไพรเมอร์ 3 ชนิด (*hmac4* *hmct1* และ *hmct5*) จากผลดังกล่าวแสดงให้เห็นว่าโซมาติกเอ็มบริโอที่ได้จากกระบวนการนี้ไม่มีความแปรปรวนทางพันธุกรรม

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ABSTRACT

Rubber tree is economically important rubber producing plant of Thailand. At present, a rubber tree plantation is susceptible to white root disease. Therefore, the use of rootstock from early introduce clone that proved to be resistant to white root disease could help sustain growing of rubber tree. Thus, the objectives of this research were to study the effects of plant growth regulators and different explants on callus and somatic embryo (SE) induction of this rubber clone. Both longitudinal thin cell layer (lTCL) from young branch and two different types of explants from young inflorescence gave 100% of sterilization and callus formation on MS medium supplemented with 2.0 mg/l 6-benzyladenine (BA) and 1.5mg/l 2,4-Dichlorophenoxyacetic acid (2,4-D) after culture for 4 weeks. The characteristics of callus from flower explant was friable and compact. Whereas the callus from lTCL was compact only. The color of callus from all sources of explants was yellowish green. For proliferation of callus, callus from mix flower gave the highest proliferation rate in terms of fresh weight at 392.05 mg after culture for 4 weeks on MS medium supplemented with the above concentrations of BA and 2,4-D. Upon transferring the callus to the same culture medium and culturing for further 12 weeks somatic embryo (SE) formation at the highest frequency of 39.84% and number of cotyledonary embryos (CEs) at 3.25 embryos /callus were obtained. CEs conversed into embryo axis at 50% and shoot at 25% after transfer to 0.25 mg/l GA_3 containing MS medium with the best concentrations of BA and 2,4-D for 4 weeks. The assessment of genetic stability of in vitro derived clones is considered to be a very

useful and essential step in this study. For SEs derived from different explants, 2 primers (OPAD-01 and OPAD-10) of RAPD and 3 primers (*hmac4 hmct1* and *hmct5*) of SSR marker gave the same profile of DNA pattern. It was clear that somaclones obtained from our protocol were uniform and successfully used to assess genetic stability in micropropagated plants.

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Kanjanee Tongtape

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LIST OF ABBREVIATIONS AND SYMBOLS

BA = 6-benzyladenine

CRD = Completely randomized design

CTAB = Hexadecyltrimethylammonium bromide

2,4-D = 2-,4-dichlorophenoxyacetic acid

DMRT = Duncan's multiple range test

DNA = Deoxyribonucleic acid

dNTP = Deoxynucleotide triphosphate

EC = Embryogenic callus

EDTA = Ethylenediaminetetraacetic acid

EtBr = Ethydium bromide

FW = Fresh weight

lTCL = longitude Thin Cell Layer

M = DNA ladder 100 bps

MS = Murashige and Skoog

NaCl = Sodium chloride

Na_2EDTA = Sodium ethylenediaminetetraacetate

PCR = Polymerase chain reaction

PGR = Plant growth regulator

PVP = Polyvinyl pyrrolidone

SE = Somatic embryo

TBE = Tris-boric acid- disodium ethylenediaminetetraacetic acid

TE = Tris EDTA

Tris = Tris (hydroxymethyl) aminomethane

CHAPTER 1
INTRODUCTION

Rationale and background

An commercially significant rubber-producing plant in the world is the rubber tree. For a century, natural rubber businesses have contributed to Thailand's socioeconomic stability (Arporn *et al*., 2010). Numerous items, including rubber tyres, medical gloves, condoms, rubber bands, flexible tubing, etc., are made using rubber latex as a raw material (Choosong *et al*., 2010). Nowadays, the pandemic of corona virus-19 causes the demand of those products from rubber, especially medical gloves, increased greatly. Therefore, expanding the area of rubber plantation to be sufficient for the industry is necessary. Grafting buds from chosen clones onto unchosen seedling rootstocks produced in polybags is the usual procedure used in the commercial production of rubber clones (Nayanakantha *et al*., 2015). 75% of Thailand's rubber-producing land is planted with the clone RRIM 600, which has been around for more than 60 years. But RRIM 600 is extremely vulnerable to illnesses brought on by Phytophthora species (Thanseem *et al*., 2005). The RRIM 600 seedling, which is mostly farmed in Thailand, is susceptible to the white-root disease, according to Wattanasilakorn *et al*. (2012). Not only does the illness reduce productivity, but it also survives for a long time on dead or living root debris. In the absence of any woody substrate, it produces many white, flattened mycelial threads that spread quickly into the soil (Nandris *et al*., 1987; Kaewchai and Soytong, 2010; Wattanasilakorn *et al*.,2012). Therefore, choosing rootstock that is resistant to white root disease can solve that problem. However, the number of rootstock is not enough to use for the demand due to the destruction of native species of rubber. Currently, the amount of native rubber trees that resistant to white root disease in Thailand has been reduced. Hence, the propagation rubber tree through tissue culture technique is necessary for increasing the number of rootstocks because it has many advantages such as the large-scale production, true-to-type and short time

required for propagation. Two techniques, somatic embryogenesis and microcutting, can be used for rubber tree micropropagation.

Axillary buds, cotyledonary nodes, or shoot tips are cultured to induce many shoots from them in the microcutting procedure. Te-chato and Muangkaewngam (1992) created numerous shoots from landrace rubber cultivars GT1 and PB5/51 *in vitro* seedlings by cultivating nodal explants. Sirisom and Te-chato (2012) reported that shoot tips derived from native cloned could be induced multiple shoot formation. Somatic embryogenesis is an effective technique for plant regeneration that complements microcutting and is crucial for transgenic methods to mass propagation and crop enhancement. Hevea's somatic embryogenesis allowed for the formation of plants from numerous explants, mostly from the inner integument (Te-chato and Chartikul, 1993; Sushamakumari *et al*., 2000; Montoro *et al*., 2003; Lardet *et al*., 2007; Kouassi *et al*., 2013) anther (Te-chato and Muangkaewngam, 1992). Hence, the objective of this study was to investigate the development an efficient *in vitro* somatic embryogenesis protocol from different parts of explant, especially floral parts of rubber tree. It is expected that floral parts should be an alternative choice for propagation of rubber tree through tissue culture technique. If the hypothesis is true this experiment will be the first report on somatic embryo induction from the floral parts of rubber tree.

CHAPTER 2
REVIEW OF LITERATURE

Importance of rubber tree (*Hevea* brasiliensis)

The tree *Hevea brasiliensis* is the most commercially significant species of the genus Hevea since its latex is the main source of natural rubber. It is a member of the Euphorbiaceae family (Anthony *et al*., 2018). A tropical tree crop, the rubber tree is primarily produced for the commercial manufacturing of latex. Similar to oil palm, it needs significant annual rainfall, little to no dry season, and constant high temperatures; the soils don't need to be particularly rich, but they do need to be deep and well drained. In many situations, oil mills and rubber treatment facilities are part of the same industrial complex since both crops are frequently cultivated in the same ecological zones (Verheye, 2010). Thailand is now the world's top producer of rubber, followed by Malaysia, Indonesia, India, Sri Lanka, and China (Yasen and Koedsin, 2015). More than 95% of the world's natural rubber comes from Southeast Asia. The tree's latex is its main commercial output, and it is obtained by slicing a small layer of the bark off the trunk. The "tapping incision" reveals the newly formed ends of the latex vessel (Chee, 1990). The rubber tree is the source of natural rubber, wood products, and rubber goods like tyres, rubber gloves, and other goods including rubber smoke sheets, block rubber, and concentrated latex. Since the 1990s, Thailand has maintained its position as the top producing nation of natural rubber. The International Rubber Study Group (IRSG) estimates that 12.9 million tons of natural rubber was produced worldwide in 2020, with the Asia-Pacific area producing 92 percent of that total. Thailand continues to be the world's top producer and exporter of natural rubber; in 2020, its production and exports totaled roughly 4.5 million tons and 38.2% of the world's total natural rubber production (Thailand Board of Investment, 2021).

White root disease

Rigidoporus microporus (Sw.) Overeem syn *R. mocroporus* (Klotzsch) Imazeki is the culprit behind the white root disease. The illness was originally noted in 1904 at Singapore's Botanical Gardens. R. microporus is a member of the Polyporaceae family's Basidiomycete order. Many commercially significant crops, including *H. brasiliensis* (Rubber tree), are affected by white root disease caused by this fungus (Kaewchai and Saitong, 2010; Mohd *et al*., 2014; Wattanasilakorn *et al*., 2017), *Artocarpus nobilis* (Ceylon breadfruit) (Madushani *et al*., 2013), *Persea americana* (Avocado) (Matinez *et al*., 2016), *Camelia sinensis* (Tea), *Mangifera indica* (Mango), *Artocarpus heterophyllus* (Jack fruit), *Ancadium occidentale* (Cashew nut), *Averrhoa carambola* (Carambola or star fruit), *Manihot esculenta* (Cassava), *Theobroma cacoa* (Cacao), *Cinnamomum verum* (Cinnamon), *Salix babylonica* (Weeping willows), *Mesue ferrea* (Na tree) (Fernandez-Fueyo *et al*, 2012), *Ficus religiosa* (Bo tree), *Acacia nilotica* (Gum Arabic tree), *Elaeis quineensis* (African oil palm), *Coffea* sp. (Coffee), *Ipomoea batatas* (Sweet potato or Yams), *Nephelium lappaceum* (Rambutan), *Solanum melongena* (Eggplant) and *Piper nigrum* (Black pepper) (Suwandi, 2003). One of the primary viruses in rubber plantations is thought to be this one. Every country that grows rubber, including West and Central Africa, India, Indonesia, Malaysia, Sri Lanka and Thailand, has white root disease. It results in bigger losses than all other illnesses and pests combined in certain nations. White root rot is caused by the fungus attacking the taproot's collar and roots. In the absence of any woody substrate, it creates many white, flattened mycelial stands that spread quickly through the soil (Nandris *et al*., 1987; Keawchai and Saitong, 2010). Contact with disease sources such rhizomorphs, diseased roots, dead stumps, or wood debris can cause the root of a healthy rubber tree to become infected (Nandris *et al*., 1987; Guyot and Flori, 2002). When that happens, a lot of trees might die and sometimes an entire stand is lost (Guyot and Flori, 2002). However, it only plays a minor part in the spread of this disease. The fruiting bodies of this fungus develop at the collar of the dead stem, which generates a huge number of basidiospores and finally kills trees at any development stage (Nandris *et al*., 1987).

All root infections are transmitted by root contact with an inoculum source, such as soil-borne infected woody debris. The disease traveled both inside and externally down the roots, causing them to decompose as it did so. Eventually, it reached the collar and tap root, where it caused the tree to quickly succumb to the disease. Dousing the soil surrounding the tree with a fungicide is a modern method of controlling the white root disease. Research has shown that using the most effective fungicides as preventative measures for rubber tree white root disease resulted in 0% infection as opposed to 95% infection in the untreated plots (Tan and Hushim, 1992; Crop Protection Research Institute, 2011).

To stop the illness from spreading to nearby trees, white root disease must be controlled immediately. Beginning disease control as soon as feasible after planting—typically approximately a year later—and doing it routinely going forward By the time the rubber trees are ready for tapping at 5–6 years old, the incidence of white root disease should have been eliminated, or at the at least, reduced. Nowadays, it is possible to manage the white root disease by combining cultural practices with chemical fungicides.

When a rubber region is to be replanted, the methods employed to remove old trees from the site define the residual level of inoculum for cultural procedures, a significant component of control. Trees are uprooted, the field is plowed and raked, and full mechanical clearing is performed in order to gather and discard the rubber roots. In a replanting region, this approach provides the lowest incidence of root disease (Newsam, 1967), yet they are pricey and cannot be used by small farmers. According to Khonglao (2006), seedlings of earliest imported clones served as the majority of the rootstock for high producing rubber plants. Wattanasilakorn *et al*. (2017) suggested that EIRpsu 5 clones generally performed well in terms of plant development while being resistant to the white root disease. Because of this, the solution to this issue is to use root stock that is resistant to white root disease.

Propagation of rubber tree

After bud grafting, rootstocks play a significant role on how well scions perform. The effectiveness of water and nutrient intake from the soil will be

influenced by plants with strong root systems (Bastiah *et al*., 1996; Soong, 1976). Additionally, it will affect how well the budded plant produces yields (Noordin *et al*., 2012). Before being suggested for large-scale planting, rubber clones should be tested for disease resistance. In addition to this genetic resilience, seedlings grown from the seeds of any earliest imported clones served as the most popular rootstock for planting material production in Thailand. Due to the transplanting of RRIM 600 clones, practically all of those clones have been lost over time. According to reports, RRIM 600, a rootstock that is extremely vulnerable to fungi that cause illnesses including phytophthora leaf fall and root rot, is used as the rootstock in around 80% of the rubber trees planted in Thailand (Crop Protection Research Institute, 2011). Therefore, resistant rootstock to those diseases must be investigated for budding with high yielding clones.

Grafting buds from chosen clones onto unchosen seedling rootstocks produced in polybags is the usual procedure used in the commercial production of rubber clones (Nayanakantha *et al*., 2015). This method maintains intra-clonal heterogeneity for both vigor and productivity (Hua *et al*., 2010). Since they were created by hybridization or selection from a small number of seedlings of Wickham germplasm, almost all of the commercially grown clones of *H. brasiliensis* reflect a relatively small genetic base (Priyadarshan and Goncalves, 2003). Due to genetic sensitivity, commercial rubber agriculture is thus always in danger of being attacked by both indigenous and invasive diseases and insects (Narayanan and Mydin, 2011). Numerous preliminary research revealed that RRIM 600 is susceptible to the white root disease and that there is no rubber clone that is resistant to it (Holiday, 1980; Nakkanong *et al*., 2008). The primary cultivar of rubber plantations in Thailand is RRIM600, and it is thought that practically all of the other early-introduced clones have been gradually lost. This suggests that there is likely a significant amount of inbreeding at the moment.

The preliminary study on this situation, which was reported by Khonglao (2006), showed that seedlings from early introduced clones had more vigorous rooting development than RRIM600 seedlings, and that it would be wise to find some other early introduced rubber clones to use as rootstock in the future. To

avoid future extinction and loss of genetic variety, it is crucial to gather and preserve such clones. White root disease poses a danger to rubber production everywhere, but is particularly severe in southern Thailand (Prasetyo *et al*., 2009). White root disease tolerance has been observed for seedlings of two rubber clones (EIRpsu1 and EIRpsu2) (Wattanasilakorn *et al*., 2012). As a result, micropropagation of rootstocks that tolerate or are resistant to root diseases is extremely important for ensuring uniform development and greatly expanding the rubber plantation.

Micropropagation of rubber tree

Today, many plant species, including rubber trees, use the tissue culture process. Micropropagation of rubber trees has been the subject of several reports. Using very little space, resources, and time, plant tissue culture (micropropagation) is a technique that enables the fast synthesis of several genetically identical plants (Odutayo *et al*., 2004). For the objective of quick multiplication, a micropropagation technique has been created. The process of *in vitro* propagation involves a number of processes, including explant selection, aseptic culture setup, multiplication, rooting, and plant adaptation. Sterilization of explants is the crucial stage in the formation of an aseptic culture. The elimination of endogenous and foreign contaminating bacteria is essential for the success of tissue culture of all plant species. (Constantine, 1986; Buckley and Reed, 1994). Various techniques have been devised to remove contamination during *in vitro* propagation (Husain *et al*., 1994). One of the most important issues facing commercial and research plant tissue facilities is *in vitro* contamination by fungus, bacteria, and yeast. Plants that have been exposed to contamination may perish or multiply less quickly. Explants must be cleaned of external pollutants such as bacteria and fungus, and it is exceedingly challenging to get sterile plant material that is fully free of contamination. When dealing with woody plant matter, it becomes more difficult (Niedz and Bausher, 2002). Surface sterilization of explants in chemical solutions is a crucial stage in the preparation process since the surfaces of living plant materials are naturally contaminated with bacteria from the environment.

A quick method to create a lot of homogeneous, genetically faithful (true-to-type plants), and healthy plantlets is plant micropropagation. There are a lot of techniques for plant micropropagation, including organogenesis and somatic embryogenesis (direct or indirect organogenesis) (Rani and Raina 2000; Beruto and Debergh 2004; Haque and Ghosh, 2016; Tisarum *et al*., 2018).

For the rubber business, microcutting is utilized to create exact replicas of planting materials. To produce plantlets, axillary buds or cotyledonary nodes are cultured (Venkatachalam *et al*., 2007). From nodal explants of in vitro seedlings GT1 and PB5/51, Te-chato and Muangkaewngam (1992) produced numerous shoots. According to the findings, the MS (Murashige and skoog, 1962) medium with BA (6-Benzyladenine) at concentrations of 4.5–5.63 mg/l produced 100% multiple shoot induction, and the mean number of shoots/explant obtained from landrace cultivars PB5/51 and GT1 were 3.0, 3.0, and 3.33 shoots, respectively. On MS medium enriched with 5.0 mg/l BA, 1.0 mg/l $AgNO_3$, and 0.5% activated charcoal, Sirisom and Te-chato (2012) cultivated shoot tips produced from native clones. According to their findings, those culture media produced the most shoots per transplant, at 5.6 shoots. Using MS media enriched with 100.0 mg/l myo-inositol, 2.0 g/l activated charcoal, 1.0 mg/l $AgNO_3$, 2.0 mg/l GA_3 (Gibberellic acid), and 5.0 mg/l KN, Anthony *et al*. (2018) generated multiple shoot from shoot tip and nodal explants (kinetin). The outcomes showed that nodal explant produced the best outcomes in terms of multiple shoot creation and quantity of shoots. 3.60 shoots were produced from each shoot tip explant, and the incidence of multiple shoot creation at the shoot tip was 84%. In the instance of nodal explants, 6.0 shoots were seen, with a 94% likelihood of multiple shoot production. In addition to these two explants, Chuaymee *et al*. (2015) reported using green budwood for the induction of numerous shoots. The maximum frequency of shoot emersion (60%) and quantity of shoots (1.6 shoots/cultured bud) were obtained in modified MS medium supplemented with 0.5 mg/l BA and 2 mg/l 2,4-D. Future rootstock propagation of this rubber tree clone will be done under these circumstances.

Due to the development of many plantlets, somatic embryogenesis is one of the most promising methods for plant multiplication (Martin, 2004). The

process of regenerating whole plants from a single somatic embryo, which itself develops from a single cell, is known as somatic embryogenesis (Komamine *et al*., 2005). Because somatic embryos are bipolar and have a lower risk of genetic abnormalities due to their single-cell origin, it is seen as superior to organogenesis (Wang and Bhalla, 2004). Somatic embryogenesis is therefore one of the effective tissue culture methods for the widespread multiplication of superior *Hevea* clones (Sirisom and Te-chato, 2013). By using this approach, a novel protocol for molecular farming through genetic formation and the mass manufacture of uniform rootstock will be made possible.

Induction and maintenance of somatic embryogenesis appear to be influenced by a number of variables, including developmental stages, explant types, concentrations of plant growth regulators and other growth substances, basal medium composition, light intensity, wound, etc. in many plants, including *Hevea*.

From immature anthers, Jayashree *et al.* (1999) created a callus, a somatic embryo, and a regenerated plant. In modified MS medium supplemented with 2.0 mg/l 2,4-D and 0.5 mg/l KN, the best callus induction results were attained. With 0.7 mg/l KN and 0.2 mg/l NAA, somatic embryo induction was shown to be more effective. On a hormone-free media, the embryos were successfully transformed into plantlets. All of the studied plantlets were shown to be diploid by cytological examination. Through somatic embryogenesis, Hau *et al*. (2010) attempted to regenerate plantlets from mature another culture (clone CATASn7-33-97 and CATAS 88-13). In modified MS medium with 4.5-13.5 µM 2, 4-D added, 85% plantlet regeneration was at its best. Then, using modified MS medium supplemented with 5.0% sucrose, 1.0 mg/l 2, 4-D, 1.0 mg/l KN, and 1.0 mg/l NAA (1-Naphthaleneacetic acid), Srichuay *et al*. (2014a) produced callus, somatic embryo, and full plantlets from anther. On modified MS medium supplemented with 3.0 percent sucrose, 0.2 mg/l NAA, 1.0 mg/l BA, 3.0 mg/l KN, and 0.05 mg/l GA_3, somatic embryo induction was achieved. The somatic embryo induction rate was 20.0% in those culture medium. Flow cytometric analysis revealed that calluses and somatic embryos from the culture media had the same ploidy level as the mother plant (Srichuay *et al*., 2014b). The best result for plantlet regeneration came from modified

MS medium supplemented with 5.0% sucrose, 0.5 mg/l KN, 0.2 mg/l BA, and 0.3 mg/l GA3. Another wall-derived calli contributed to the success of embryoid and plant development through somatic embryogenesis (Wang *et al*., 1984; Jayashree *et al*., 1999; Srichuay *et al*., 2014b) the somatic tissue of which. Plantlets produced through this explant culture have therefore been shown to be diploid and have the same genetic makeup as the mother plant. The ideal procedure for callus induction, somatic embryogenesis, and plant root regeneration was described by Zhou *et al*. (2010). In MS medium supplemented with 1.0 mg/l KN and 0.2 mg/l BA, maximum callus induction was achieved. On such culture medium, somatic embryo induction and embryonic development into plantlets were achieved after a 12-week culture period. Te-chato and Chartikul (1993) used modified MS medium enriched with 5.0-6.0 percent sucrose, 2.0 mg/l 2,4-D, 2.0 mg/l BA, and adjusted pH to 5.6-5.8 to successfully promote embryogenic callus and plantlets regeneration from inner integument culture of immature fruit (8 weeks after pollination). Excision of each embryoid 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 media improved maturation and germination of these embryoids. A method for modified MS medium enriched with 0.90 µM 2, 4-D, 2.68 µM NAA, 0.93 µM KN, 3.0% sucrose, and 0.2% phytagel was developed by Sushamakumari *et al*. in 2000. On a modified MS medium containing 370 mg/l KH_2PO_4, 120 mg/l $MgSO_4.7H_2O$, 1.33 µM BA, 1.07 µM NAA, 3.0% sucrose, and 0.2% phytagel, somatic embryo induction was discovered. On this medium, continued growth of the embryo into plantlets was accomplished as previously mentioned. On MH medium supplemented with 4.5 µM 3, 4-D (3, 4-Dichiorophenoxyacetic acid), 4.5 µM KN, 30 µM $AgNO_3$, 12.0 µM $CaCl_2$, and 234 mM sucrose, Montoro *et al*. (2003) and Lardet *et al*. (2007) also succeeded in inducing embryogenic callus from inner integument culture (clone PB260). Additionally, a number of studies attempted to produce somatic embryos from different *Hevea* explants. Kongkaew (2017) created a methodology for green bud callus induction and plant regeneration. The maximum callus production at 100% came from green buds from *ex vitro* produced plants that were sterilized and kept at 40°C for 24 hours. They were subsequently cultivated on modified MS medium

supplemented with 2 mg/l 2,4-D and 0.5 mg/l BA for 6 months. After two months of culture, the medium enriched with 1.5 mg/l $AgNO_3$ produced the highest fresh callus weight at 950 mg and the maximum number of somatic embryos per tube at 9.1. The number of globular embryos produced, at 11.61, and the number of cotyledonary embryos, at 18.4, were all maximum during the second round of culturing (81.33 percent). The maximum plant regeneration was achieved at 40% following subculture of cotyledonary embryo to PGR-free MS medium and one month of culture. the typical number of whole plantlets per tube (0.24 plants). Using MS media enriched with 2 mg/L 2,4-D and 0.5 mg/L BA, Kongkaew et al. (2016) created a callus from a longitudinally thin cell layer (lTCL) in the distal region of the culture. After 17 days of culture, the results showed that the culture media had the greatest callus induction rate, at 67.5%. But no effective method for mass micropropagation of superior *Hevea* clones has yet been created (Nayanakantha and Seneviratne, 2007).

Assessment of genetic stability

In nature, recombination processes produce the genetic variety and variability within a population. Genetic diversity is influenced in many ways by factors including natural selection, mutation, migration, and population size. Plants grown from any type of cell culture were referred to as "somaclones," and the genetic variation seen in such plants was referred to as "somaclonal variation." Asexual processes, which solely include mitotic division of the cells, are used to develop plant cells *in vitro* and regenerate them into whole plants. When cultivating plant tissue, the incidence of unplanned and random spontaneous variation is less of a concern (Skirvin *et al*., 1994). The primary goal of tissue culture is to produce true-to-type plants in order to preserve the germplasm, although there is a possibility of genetic aberration, also known as "somaclonal variants," during tissue culture (Arvind *et al*., 2015). Regenerated plants formed from organ cultures, calli, protoplasts, and somatic embryos can occasionally display phenotypic and genotypic diversity due to the potentially mutagenic nature of *in vitro* growing conditions (Leva *et al*., 2012). However, it has been noted that the tissue culture environment and the effects of

culture conditions, such as culture media, types of explants, successive transfers of culture, temperature, pH, plant growth regulators, etc., cause changes in cellular controls and lead to genomic changes in the *in vitro* raised plantlets (Rani and Raina 2000; Jain, 2001; Manoj *et al*., 2012). A typical method of producing somaclonal diversity is the callus induction and subsequent plant regeneration from callus culture (Skirvin *et al*., 1994; Anandan *et al*., 2018). Somaclonal variation in regenerants will prevent more widespread economic exploitation of crop species. Therefore, it is essential to maintain the *in vitro* grown plantlets' true-to-type characteristics with regard to the mother plant (Ananda *et al*., 2018). In order to prevent these changes, it is always important to evaluate the genetic integrity of *in vitro* produced plants. Molecular markers, which are DNA-based markers important for detecting genetic similarities in micropropagated tree species, are the technique that is most frequently utilized in diverse laboratories. Environmental and biological elements have no impact on this method (Manisha *et al*., 2020).

Recent research has shown that polyploidy, aneuploidy, chromosomal breakage, deletion, translocation, gene amplification, and mutations are prevalent genetic alterations in cell or tissue cultures. These changes are also manifested at the biochemical or molecular levels (Teixeira *et al*., 2007). In plant tissue culture and regenerants of various plants, somaclonal variation has been highlighted using various molecular analytical approaches. RAPD (randomly amplified polymorphic DNA) and SSR (simple sequence repeat) are frequently utilized in studies of genetic diversity in regenerated plants from tissue culture, such as sesame (Anandan *et al*., 2018), chili pepper (Tilahun *et al*., 2020), plum (Manisha *et al*., 2020) stevia (Veronica *et al*., 2021) and rubber tree (Feng *et al*., 2014; Sirisom and Te-chato, 2014).

Following biochemical markers such as isozymes (Chevallier, 1998), molecular markers have been developed and used since the middle of 1990’s for diversity studies (Besse *et al*., 1994; Le Guen *et al*., 2011), genetic mapping (Lespinasse *et al*., 2000) and the identification of genetic loci implicated in the expression of agronomic traits in *H. brasiliensis* (Lespinasse *et al*., 2000; Le Guen *et al*., 2011). In the recent past, the molecular markers have been helpful tools for studying the genetics of *H. brasiliensis* (Sirisom and Te-chato, 2014). Hevea breeding

has used molecular tools and markers over the past 20 years (Venkatachalam *et al*., 2007; Sirisom and Te-chato, 2014). With *H. brasiliensis*, many molecular marker techniques have been employed. For instance, Besse *et al*. (1994) used RFLP analysis to examine the genetic diversity of 92 Amazonian and 73 Wickham clones. For the purpose of identifying offspring with two common parents, Low *et al*. (1996) also employed RFLPs. Sirisom and Te-chato (2014) used SSR markers to evaluate the genetic integrity of the nodal culture *in vitro* of early imported clones of rubber plants. By analyzing the genetic connections between the early-introduced clone populations and RRIM 600, Wattanasilakorn *et al*. (2015) were able to estimate the polymorphism of DNA fragment patterns from those populations. When these several marker systems are used together, a more accurate analysis is produced than when they are used alone (Pethin *et al*., 2015).

The most popular method of genetic mapping is now SSRs based on straightforward PCR tests due to its benefits of high polymorphism, co-dominance, specificity, wide dispersion, low cost (Lan *et al*., 2012), germplasm identity (Ye *et al*., 2009; Jia *et al*., 2014), gene localization, molecular marker–assisted selection breeding and genetic diversity analysis (Bonierbale *et al*., 1988; Powell *et al*., 1996; Jones *et al*., 1997; Song *et all*., 2016). 53 early imported rubber tree clones were gathered from various southern Thailand regions and examined by Nakkanong *et al*. in 2008. Microsatellite markers were used to operate the assessment. In all, 44 amplified fragments were generated by four microsatellite primer pairs (hmac4, hmct1, hmct5, and hmac5), with an average of 14.67 fragments per primer, according to the data. Only hmac5 yielded monomorphic fragments, whereas 37 of those primers were polymorphic (84.09%). Through SSR analysis, Sirisom and Te-chato (2014) evaluated the genetic integrity of *in vitro* numerous shoots produced from the nodal cultures of early imported rubber tree clones. The three SSR marker primers (hmac4, hmct1, and hmct5) used to create *in vitro* plantlets displayed identical DNA pattern profiles.

The RAPD methodology was recommended by Varghese *et al*. (1997) as a useful tool for identifying rubber trees. By using the RAPD approach, Sirisom and Te-chato (2013) evaluated the genetic integrity of in vitro rubber tree plantlets. Five

primers (OPAD-01, OPAD-10, OPAD-12, OPAB-17, and OPR-02) were found to be able to amplify and provide monomorphic DNA patterns among *in vitro* rubber plantlets, according to the results. Using seven RAPD primers, Srichuay and Te-chato (2014) examined the somaclonal variation of an *in vitro* rubber tree produced from callus and somatic embryo (OPAB-01, OPAD-01, OPAD-10, OPB-17, OPN-16, OPR-02 and OPZ-04). According to the findings, OPZ-04, one of the seven primers, produced polymorphism in the DNA profiles of the materials examined. Using 10 primers for RAPD markers, Wattanasilakorn *et al*. (2015) compared genetic analysis of EIRpsu 1 and EIRpsu 2 to RRIM 600 (the main *Hevea* rubber cultivar in Thailand) (OPR-02, OPR-11, OPAD-01, OPAD-10, OPAD-12, OPB-12, OPB-17, OPR-02, OPZ-04, OPN-08 and OPC-05). According to the results of the cluster analysis, EIRpsu 2 was more similar to RRIM 600 than EIRpsu 1. In order to demonstrate the intimate relationship between rootstock and scion, it is crucial to analyze the genetic fidelity of rubber tree rootstock generated from tissue culture.

Objectives

1. To study the factors affecting induction of callus from different types of explants of *Hevea*
2. *To factors affecting induction of callus of SE from different explants*
2. To regenerate SE from callus induced from different types of explants of *Hevea*
3. To evaluate the genetic stability of SE of *Hevea* using RAPD and SSR markers

CHAPTER 3
MATERIALS AND METHODS

1. Materials and equipments

1.1 Plant materials

- Young branches and Young inflorescences of early introduced clone of rubber tree (EIRpsul) grow naturally at Prince of Songkla University, Hatyai campus, Songkhla province, Thailand, were use in this experiment. The early introduced rubber clone were assessed resistant to white root disease (Wattanasilakorn *et al*., 2017).

1.2 Chemicals

- Chemicals used for MS medium formulas (appendices)
- Chemicals for adjusting pH
    - HCl
    - KOH
- Plant growth regulators
    - 2, 4-D
    - BA
    - GA_3
- Surfactant
    - NaOCl (Sodium hypoclorite)

- Ethanol
- Tween-20

- Chemicals for DNA extraction
  - CTAB (Hexadecyl trimethyl-ammonium bromide)
  - β-mercaptoethanol
  - PVP-40 (Polyvinyl pyrrolidone)
  - NaCl (Sodium chloride)
  - Na_2EDTA (Ethylenediaminetetraacetic acid disodium salt dihydrate)
  - Chloroform
  - Isopropanol
  - TE buffer
  - Ethanol
- Chemicals for electrophoresis
  - Agarose
  - H_3BO_3 (Boric acid)
  - Tris-base
  - Ethidium bromide
  - Lamda DNA (λ DNA)

- 100 bp and 500 bp DNA Ladder (Operon, U.S.A.)

- Chemicals for PCR
  - dNTP (dATP, dTTP, dCTP and dGTP) (Promega, USA)
  - ISSR primers
  - RAPD primers
  - $MgCl_2$
  - *Taq* DNA Polymerase B (Promega, USA)
  - 10X *Taq* buffer

1.3 Equipments

- For culture medium preparation
  - 2 and 4 decimal balances
  - pH meter
  - Autoclave
  - Drying and sterilizing cabinet
  - Refrigerator and freezer
  - Glassware such as tubes, pipettes, volumetric flask
- For culturing explant
  - Forceps, , blade, blade holder

- Laminar air flow

- For DNA extraction, electrophoresis and PCR technique
  - Vortex mixer
  - Microcentrifuge
  - Micro centrifuge tube
  - Micro pipette
  - Stirrer
  - Electrophoretic equipments
  - DNA amplifier machine
  - Microwave oven
  - Gel documentation system
  - Nanodrop spectrophotometer

2. Methods

2.1 Effects of types of explants and concentrations of sodium hypochlorite on sterilization and callus induction

Explants used in this experiment divided into two different sources, young branch (Figure 1a) and young inflorescence (Figure 1b). Those explants were taken from rubber tree clone EIRpsu I, resistant to white root disease (according to Wattanasilakorn *et al*., 2017) at the Faculty of Natural Resources, Prince of Songkla

University, Songkhla**,** Thailand. Both explants were washed with running tap water for 20-30 min, surface sterilized in 70% ethanol for 30 second and soaked in different concentrations of NaOCl; 0, 0.2625, 0.5250, 0.7875 and 1.0500% for 20 min, followed by rinsing three times with sterilized distilled water. For young branch sterilized explants longitudinal thin cell layer (lTCL) at length of 5 mm were excised from its internode (Figure 2a). In case of inflorescence, petals from individual male flower were excised under stereo microscope (Figure 2b). The petals, single flower and mix flowers were chopped with sharp lazor blade for 20 times (Figure 2c) and mix flowers (3 flowers) were chopped for 60 times (Figure 2d). The explants were cultured on MS medium supplemented with 2.0 mg/l BA and 1.5 mg/l 2,4-D. The pH of the culture medium was adjusted to 5.7 with 1.0 N KOH before adding 0.7% agar and autoclaving at 1.05 kg/cm^{2}at 121°C for 15 min. The cultures were maintained at 28 ± 2°C under fluorescent bulbs at 15.0 µmol/m^2/s for 14 hour photoperiod. After one month of culture the percentage of contamination and callus induction were recorded.

Figure 1 Young branch (a) and young inflorescence (b) of rubber tree clone EIRpsul used for sterilization as initial explants for callus induction

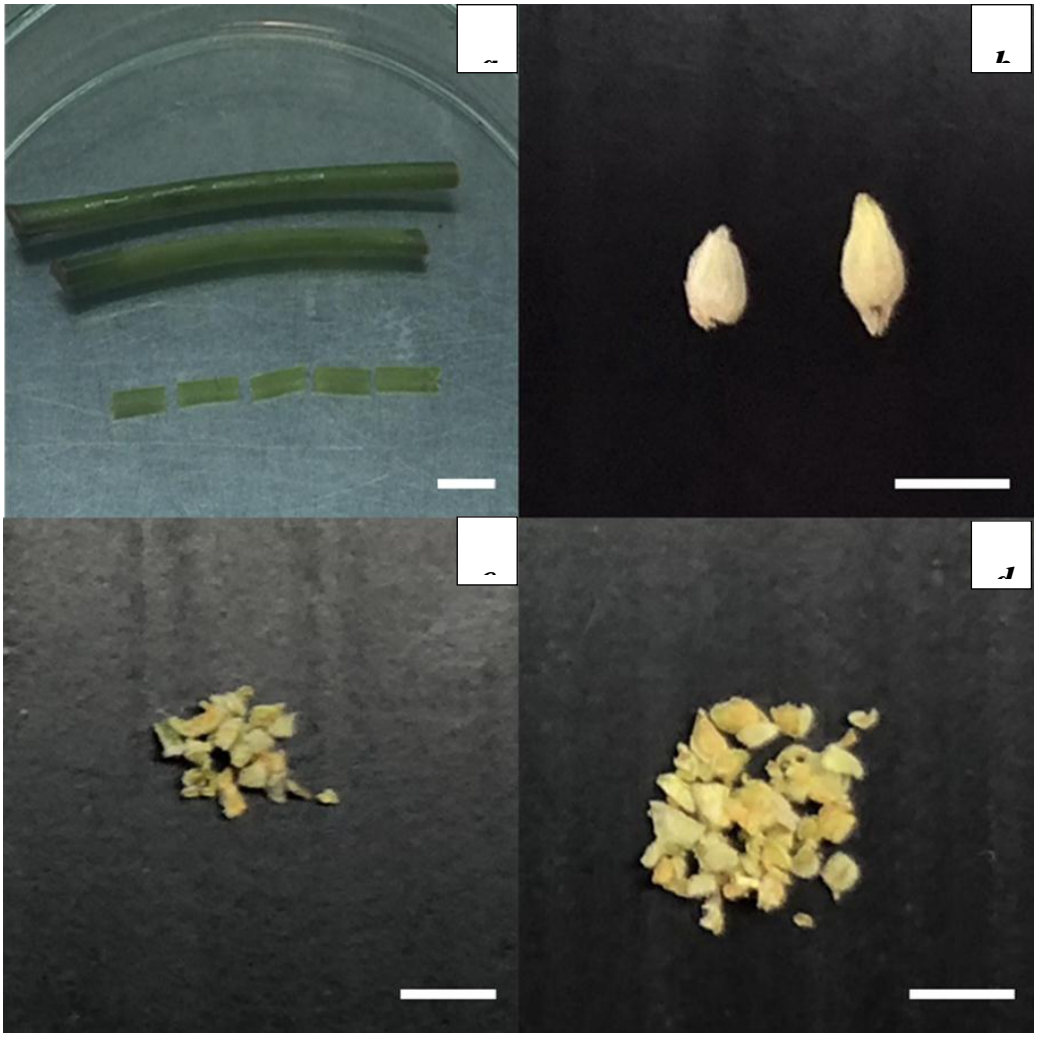

Figure 2 Preparation of each explant types of rubber tree for culturing on MS medium with MS medium supplemented with 2 mg/l BA and 1.5 mg/l 2,4-D for callus induction

a: lTCL (bar=0.5 cm)

b: Petal (bar=0.2 cm)

c: Chopped single flower (bar=0.2 cm)

d: Chopped mix flowers (bar=0.2 cm)

2.2 Effects of types of explants and plant growth regulators on callus induction

The sterilized explants from experiment 1 consisted of lTCL from internode of young branch, chopped petal, single flower and mix flowers were cultured on MS medium supplemented with BA at different concentrations (0, 0.5, 1.0, 1.5 and 2.0 mg/l) in combination with 2, 4-D at different concentrations (0, 0.5, 1.0, 1.5 and 2.0 mg/l). The pH of the culture medium was adjusted to 5.7 with 1.0 N KOH before adding 0.7% agar and autoclaving at 1.05 kg/cm^{2}and 121°C for 15 min. The cultures were maintained at 28 ± 2°C under fluorescent bulbs at 15.0 µmol/m^2/s for 14 hour photoperiod. After one month of culture the percentage of callus induction and characteristics of callus were recorded. Factorial in completely randomized design (CRD) was performed. Callus obtained from each types of explants and concentrations of plant growth regulators was statistically analyzed using ANOVA and means among treatments and treatment combinations were separated by Dancan's multiple range test (DMRT).

2.3 Effects of types of explants and number of chopping on callus fresh weigh

Callus derived from lTCL, petal, single flower and mix flower from experiment 2.2 at 100 mg was wounded by different numbers of chopping at 0, 50, 100, 150 and 200 times and cultured on MS medium supplemented with the best concentration of BA and 2, 4-D from experiment 2.2. The pH of the medium was adjusted to 5.7 with 1.0 N KOH before adding 0.7% agar and autoclaving at 1.05 kg/cm^{2}and 121°C for 15 min. The cultures were maintained at 28 ± 2°C under fluorescent bulbs at 15.0 µmol/m^2/s for 14 hours photoperiod. After one month of culture the callus fresh weigh was recorded and compared using factorial in CRD.,

The data were statistically analyzed using ANOVA and means among the treatments and treatment combinations were separated by DMRT.

2.4 Effect of types of explants on somatic embryo (SE) formation

The callus derived from different types of explants (lTCL, petal, single flower and mix flower) from the best number of chopping in experiment 2.3 were cultured on MS medium supplemented with the best concentration of BA and 2, 4-D. The pH of the medium was adjusted to 5.7 with 1.0 N KOH before adding 0.7% agar and autoclaving at 1.05 kg/cm^{2}and 121°C for 15 min. The cultures were maintained at 28 ± 2°C under fluorescent bulbs at 15.0 µmol/m^2/s for 14 hours photoperiod. The cultures were sub-cultured every 4 weeks for 12 weeks. After 3 months of culture the percentage of SE formation and number of SEs/explant were recorded. The data were statistically analyzed using ANOVA and the means among the treatments were separated by DMRT.

2.5 Effects of types of explants and GA_3 on development of SE

SEs at cotyledonary stage derived from experiment 2.4 were cultured on MS medium supplemented with GA_3 at different concentrations (0, 0.25, 0.50, 0.75 and 1.00 mg/l) and the best concentration of BA and 2, 4-D from experiment 2.4. The pH of the medium was adjusted to 5.7 with 1.0 N KOH before adding 0.7% agar and autoclaving at 1.05 kg/cm2 and 121°C for 15 min. The cultures were maintained at 28 ± 2°C under fluorescent bulbs at 15.0 µmol/m^2/s for 14 hours photoperiod. After one month of cultured the percentage of plant regeneration was recorded. Factorial in completely randomized design (CRD) was performed. Compared in each type of explants and concentrations of GA_3, the data were statistically analyzed using ANOVA and the means among the treatments and treatment combinations were separated by DMRT.

2.6 Assessment genetic stability

2.6.1 DNA extraction

Young fully expanded leaves from mother plant (MP) of rubber tree (EIRpsuI) and SE from *in vitro* plantlet derived from different explant (S1-3 = petal, S4-6 = single flower and S7-9 = mix flowers) at 100 mg were used for DNA extraction according to the procedure modified from Doyle and Doyle (1990). Briefly stated, DNA was extracted from the explants using CTAB extraction buffer, which contains 2 percent hexadecyltrimethyl-ammonium bromide (CTAB), 20 mM EDTA, 100 mM Tris-HCL pH 8.0, and 1.4 M NaCl. The plant extract mixtures were transferred to the microcentrifuge tube, incubated for 45 minutes at 60°C in a water bath, and then centrifuged for 45 minutes at 10,000 rpm. Onto a fresh new microcentrifuge tube, the supernatant was transferred. 500 ml of chloroform was added to each tube, the solution was mixed by inverting the tube, it was centrifuged at 12,000 rpm for 10 minutes, and the top aqueous phase alone (which contains the DNA) was transferred to a clean microcentrifuge tube. To precipitate the DNA, 750 µl of isopropanol was added to the solution, and the tube was slowly inverted multiple times. Following precipitation, the DNA pellet was thrice rinsed with 70% ethanol before being allowed to air dry. The quantity of isolated DNA were determined by nanodrop spectrophotometer before dissolving in TE buffer [20 mM Tris-HCL (pH 8.0) and 0.1 M EDTA (pH 8.0)] and stored at 4°C for further use in polymerase chain reaction (PCR) analysis.

2.6.2 RAPD analysis

RAPD analysis was operated according to the methodology of Nakkanong *et al.* (2008); Sirisom and Te-chato (2013b). 25 mM $MgCl_2$, 10x *Taq* buffer, 100 M of each dNTP, 0.3 mM of each primer (OPAD01 and OPAD10), 1.5 units of Taq polymerase, and 60 ng of template DNA were all included in each 25 µl amplification mixture. For RAPD-PCR, the heat profile was 95°C for 30 seconds, then 41 cycles of 37°C for 1 minute, 72°C for 2 minutes, and lastly 72°C for 5 minutes. Following electrophoresis on 1.7 percent (w/v) agarose gel in 0.5x Tris-borate-EDTA (TBE) buffer at constant 100 V for 35 minutes, amplification products were separated. The gels were exposed to ultraviolet light with gel documentation after 15 minutes of

ethidium bromide staining, 5 minutes of immersion in distilled water. Photographs of the DNA amplification products from various sample sources and mother plants were compared.

2.6.3 SSR analysis

A PCR procedure reported by Thawaro and Te-chato (2009) was utilized to amplify DNA using three SRR primer pairs (hmct5, hmac4, and hmtc1). 2.5 mM MgCl2, 10x *Taq* buffer, 100 M of each dNTP, 0.3 mM of primer, 1.5 units of Taq polymerase, and 20 ng of template DNA were all included in each 10 µl amplification mixture. The PCR amplifications were carried out using a thermocycler (TC-XP-G, Japan). A 30-second denaturation phase was followed by 34 cycles of 95°C for 30 seconds, 52°C for 60 seconds, and 62°C for 120 seconds. The last 5-minute elongation step was performed at 72°C. On a 3% (w/v) agarose gel, in 0.5x TBE buffer, at constant 100 V for 55 minutes, DNA products were separated. The gels were exposed to ultraviolet light with gel documentation after 15 minutes of ethidium bromide staining, 5 minutes of immersion in distilled water. Between samples and mother plants, the DNA amplification products were compared.

CHAPTER 4
RESULTS AND DISCUSSIONS

1. Effects of types of explants and concentrations of sodium hypochlorite on sterilization

All of explants gave percentage of clean culture at 100 when treated with NaOCl at concentrations 0.5250, 0.7875 and 1.05% (Figure 3). These results are in accordance with the experiments of Badoni and Chauhan (2010) who reported that NaOCl at 1.05% was the best concentration for controlling the infection of potato cv. 'Kufri Himalini'in tissue culture processes. However, Altaf (2006) reported that slightly high concentration of NaOCl at 1.3125% were effective in making clean explants of kinnow tree. It has been shown that sodium hypochlorite is highly efficient against a variety of bacteria; even micromolar doses are sufficient to dramatically lower bacterial populations (Nakagawara *et al*., 1998). For callus induction in this present study, NaOCl at only concentration of 0.2625 and 0.5250% gave the highest result at 100% in all explants (petal, single flower, mix flower and lTCL). However, increase in concentrations of NaOCl from 0.7875 to 1.05% in all explants caused the decrease in percentage callus induction from 68 to 60%, (Figure 4). Additionally, it has been noted that the hypochlorite salts [NaOCl, $Ca(OCl)_2$, LiOCl, and KOCl] diluted with water produced HClO, which had a negative correlation with bactericidal activity, perhaps because it caused fatal DNA damage. (Wlodkowski and Rosenkranz, 1975; Dukan *et al*., 1999). According to Ines *et al*. (2013), human error-related damages sustained during the sterilizing procedure and meristem isolation caused some of the experiment's explants to not survive. This can be explained by the fact that various tissue types and the characteristics of the explant utilized for micropropagation have varying needs for sterilizing.

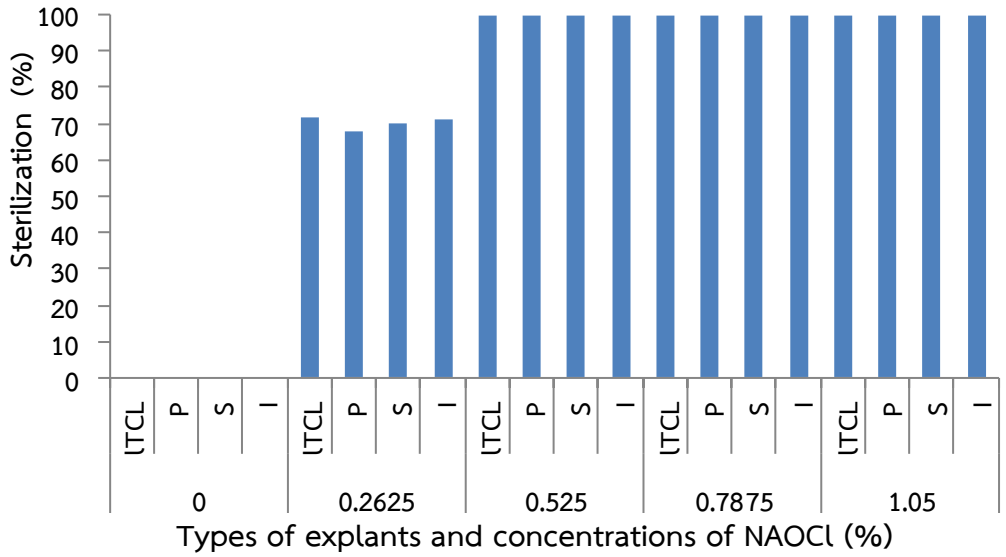

Figure 3 Influence of types of explants and concentrations of NaOCl on sterilization of *Hevea* cultured on MS medium with 2.0 mg/l BA and 1.5 mg/l 2,4-D for 4 weeks

lTCL: Longitude thin cell layer, P: Petal S: Single flower and I: Mix flower

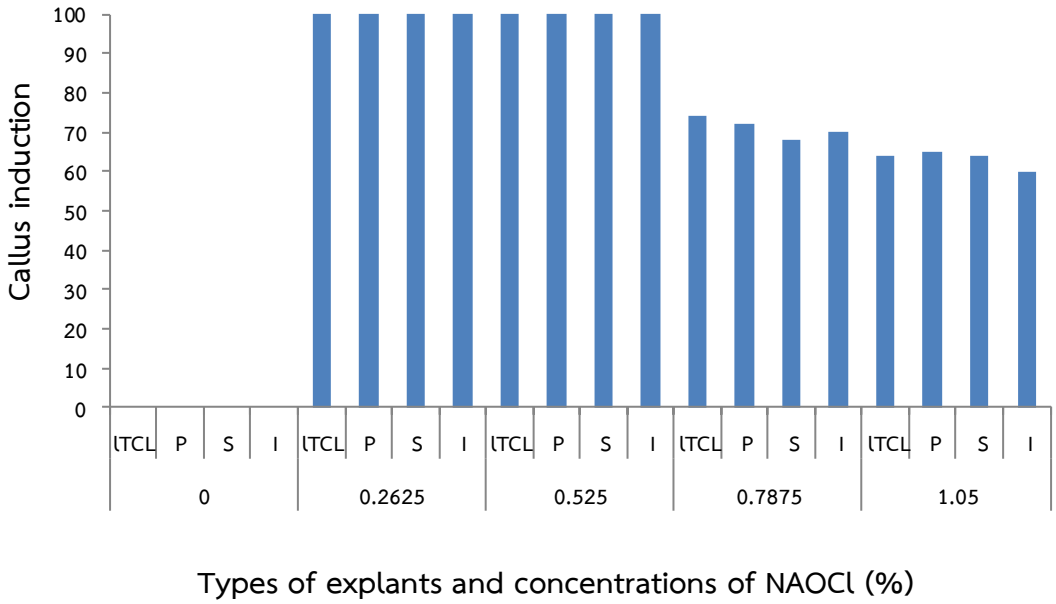

Figure 4 Influence of kinds of explants and concentrations of NaOCl on callus induction of *Hevea* cultured on MS medium with 2.0 mg/l BA and 1.5 mg/l 2,4-D for 4 weeks

lTCL: Longitude thin cell layer, P: Petal S: Single flower and I: Mix flower

2. Effects of types of explants and plant growth regulators on callus induction

Explants, PGRs, and culture conditions are only a few of the variables that determine how callus is induced in plants. PGRs are one of those that are important for both the induction and proliferation of callus. Additionally, several researches have noted that varying PGR doses and combinations significantly affect the formation of callus (Poeaim *et al*., 2005; Sun *et al*., 2006). It was clearly showed that plant growth regulators both kind and concentrations play different roles in callus induction from different types of explants (lTCL, petal, single flower and mix

flower). Therefore, both factors (PGRs and explant types) are important in induction of callus. The result showed that the highest percentage of callus induction at 100 was obtained from lTCL, petal and mix flowers when those explants were cultured on MS medium supplemented with 2.0 mg/l BA and 1.5 mg/l 2,4-D, significantly different with another treatments (Table 1). Low concentration of 2,4-D containing MS medium in the present study enhanced the ability of induction of callus. Several writers have claimed that a low concentration of this PGR was beneficial for the development of embryogenic callus from *Panax ginseng* roots (Chang and Hsing, 1980) and *Lycium barbarum* (Hu *et al*., 2008). Whereas Wang *et al*. (2006) found that high concentrations of 2,4-D promoted callus induction in *Areca catechu*. Moreover, the use of 2,4-D in combination with BA could promote callus induction from lTCL of *Hevea brasiliensis* (Kongkaew *et al*., 2016). In case of types of the explants, only lTCL provided compact yellow callus (Figure 5a) whereas the callus derived from single flower, petal and mix flowers was compact green, friable and compact yellow, respectively (Figure 5b, c, and d). Farhadi *et al.* (2017) reported the culture of different explants of *Allium hirtifolium* that the explants of basal plate showed up to 60.06% callus formation after 12 weeks and leaves exhibited a significantly lower callus induction up to 43.68%. Thus, basal plate was better explant for callus production. Therefore, the explant type, concentrations and combinations of plant growth regulators are the most important factors affecting callus induction (Scotton *et al*., 2013).

Table 1 Effects of types of explants and PGRs containing MS medium on callus induction after 4 weeks of culture

2,4-D	BA	Callus induction (%) from different types of explants				Characteristic of callus			
mg/l		lTCl	P	S	M	lTCl	P	S	M
0	0	-	-	-	-	-	-	-	-
0.5	0.5	0	12.54^{i}	15.60^{hi}	20.50^{h}	0	FC	CC, FC	CC, FC
	1.0	0	12.75^{i}	17.33^{h}	20.68^{h}	0	FC	CC, FC	CC, FC
	1.5	10.33^{h}	18.45^{h}	24.21^{g}	30.65^{g}	CC	CC, FC	CC, FC	CC, FC
	2.0	27.84^{gh}	20.33^{h}	14.78^{i}	52.82^{f}	CC	CC, FC	CC, FC	CC, FC
1.0	0.5	34.6^{g}	40.62^{g}	39.17^{f}	53.40^{f}	CC	FC	CC, FC	CC, FC
	1.0	25.34^{gh}	45.63^{fg}	55.67^{de}	60.33^{e}	CC	CC, FC	CC, FC	CC, FC
	1.5	31.28^{g}	50.24^{f}	60.33^{d}	71.84^{d}	CC	CC, FC	CC, FC	CC, FC
	2.0	40.63^{f}	42.78^{fg}	62.45^{d}	70.62^{d}	CC	FC	CC, FC	CC, FC
1.5	0.5	40.69^{f}	70.33^{d}	63.90^{d}	80.43^{c}	CC	CC, FC	CC, FC	CC, FC
	1.0	54.87^{d}	80.15^{c}	79.46^{bc}	89.40^{bc}	CC	CC, FC	CC, FC	CC, FC
	1.5	70.59^{c}	98.18^{b}	84.84^{b}	96.56^{b}	CC	CC, FC	CC, FC	CC, FC
	2.0	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}	CC	CC, FC	CC, FC	CC, FC
2.0	0.5	85.33^{b}	64.50^{de}	84.67^{b}	85.43^{bc}	CC	CC, FC	CC, FC	CC, FC
	1.0	54.52^{d}	62.83^{de}	74.22^{c}	80.45^{c}	CC	CC, FC	CC, FC	CC, FC
	1.5	52.44^{de}	54.72^{f}	52.74^{e}	60.84^{e}	CC	CC, FC	CC, FC	CC, FC
	2.0	40.64^{f}	60.18^{de}	40.15^{f}	50.62^{f}	CC	CC, FC	CC, FC	CC, FC
F-test			**						
C.V. (%)			54.62						

lTCL; Longitude thin cell layer, P; Petal, S; Single flower, M; mix flower, CC; Compact callus, FC; Friable callus ** = significant different at P ≤ 0.01

Means followed by the same letter within column are not significantly different according to DMRT

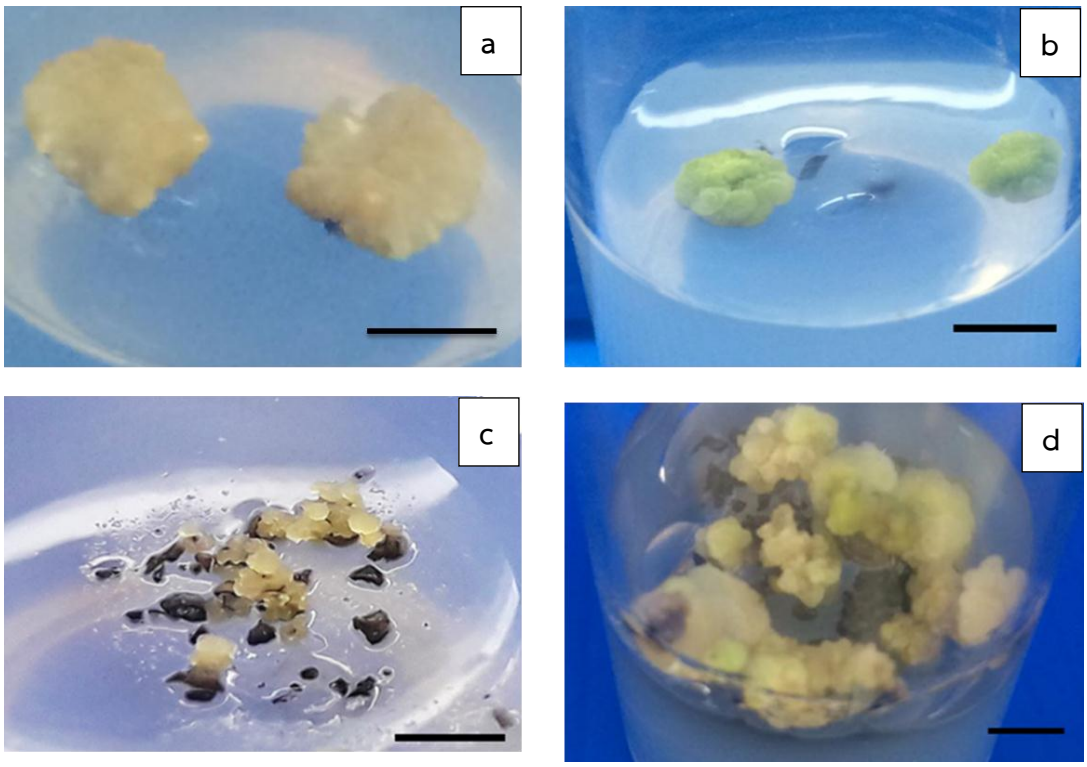

Figure 5 Morphological characteristics of callus induced from different explant types on MS medium supplemented with 2.0 mg/l BA and 1.5 mg/l 2,4-D after 4 weeks of culture (bars= 0.5 cm).

a: lTCL **b**: petal **c:** single flower **d:** mix flowers

3. Effects of types of explants and number of chopping on callus fresh weight

In the present study, the callus derived from different types of explants (lTCL, petal, single flower and mix flower) was wounded through chopping for 0, 50, 100, 150 and 200 times cultured on MS medium supplemented with the best concentration of BA and 2, 4-D from previous experiment for 4 weeks. The results revealed that callus derived from petal, single flower and mix flower chopped at 100 times gave the best result in proliferation. Callus fresh weight obtained from those explants were 385, 387 and 392 mg, respectively (Figure 6), significantly different with another treatments. For the characteristic of callus from those explants was friable and yellow color (Figure 7). However, the callus derived from lTCL could not increase fresh weight in all numbers of chopping. This result was similar to the

study of Kongkeaw (2017) who reported that chopping the callus of *Hevea* for 100 times gave the best result in proliferation of callus in term of increase in fresh weight after 4 weeks of culture. Tongtape and Te-chato (2010) observed that chopping of oil palm callus affected both proliferation and initiation of embryogenic callus. Djibril *et al*. (2012) reported that the secondary friable calli of date palm obtained from chopped granular calli could initiate embryogenic cell suspension. Chopping is a method to produce the wound and encourage callus to increase drastically. In accordance with Sidky and Gadalla (2013), they reported that chopped friable callus of *Phoenix dactylifera* could increase the number of calli. Moreover, the formation of wounds is another way to increase the efficiency in absorbing water and minerals. However, the callus from lTCL had less effective through this method due to the callus was compact and green color. When it was chopped browning occurred due to the production of phenolic compounds leading to dead of callus tissues finally.

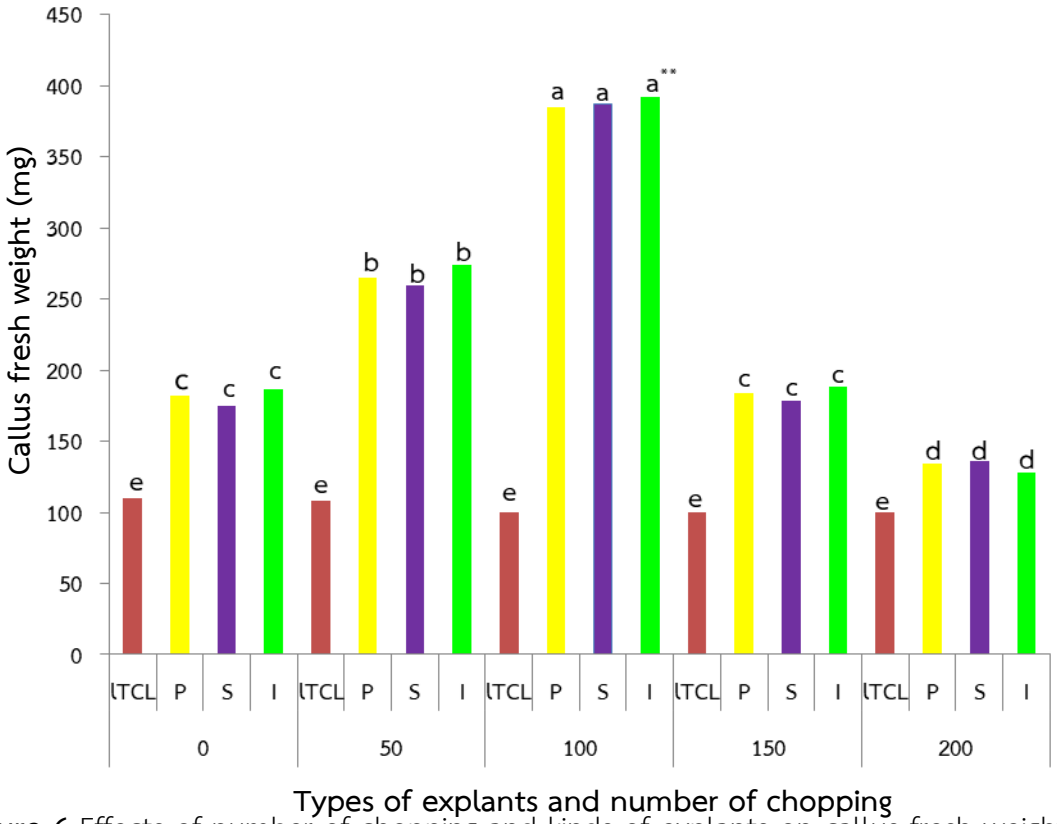

Figure 6 Effects of number of chopping and kinds of explants on callus fresh weight on MS medium supplemented with 2.0 mg/l BA and 1.5 mg/l 2,4-D after 4 weeks of culture

** = significantly different ($P \leq 0.01$)

Means followed by the same letter within histogram are not significantly different according to DMRT

lTCL: Longitude thin cell layer, P: Petal S: Single flower and I: Mix flower

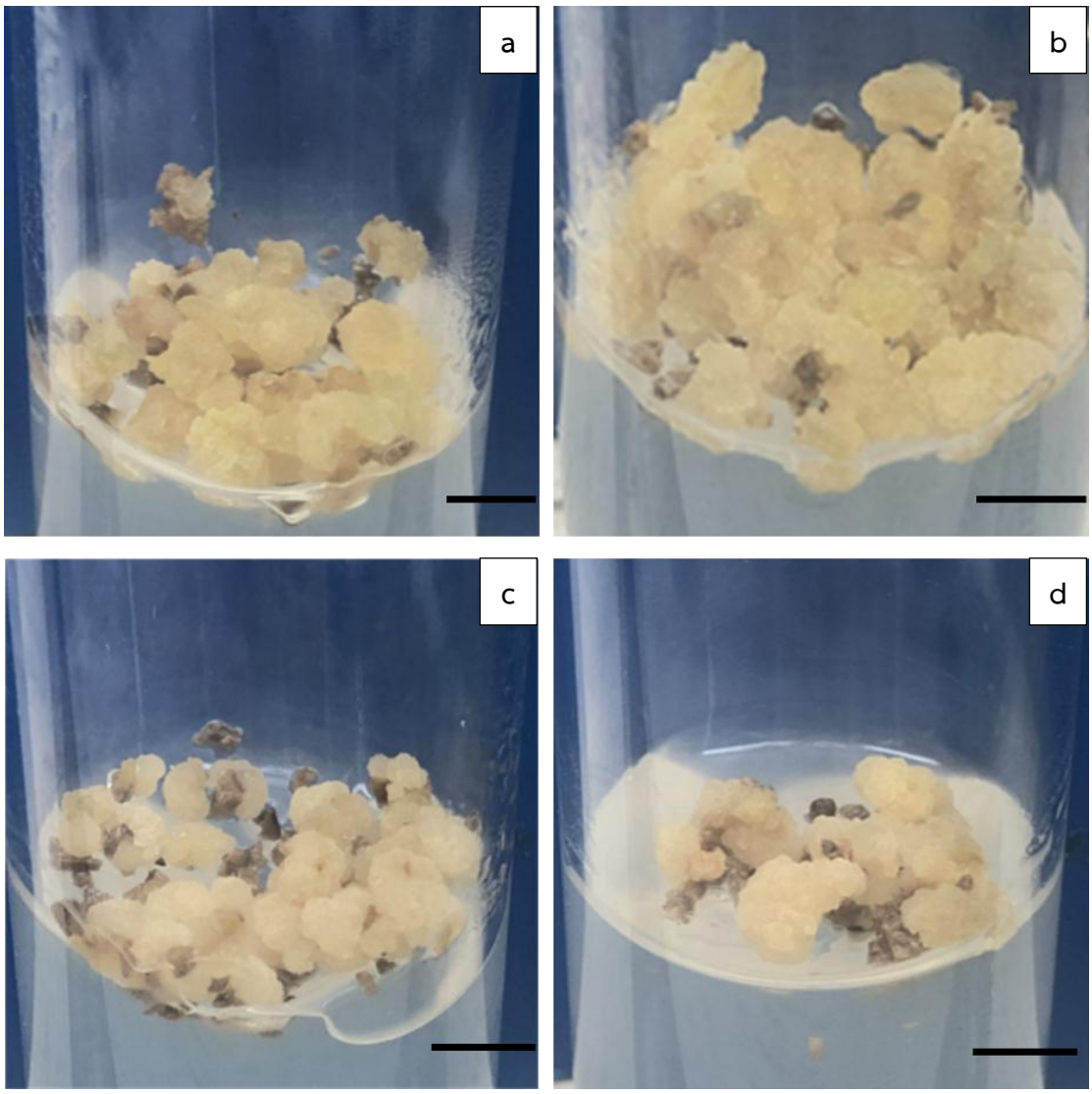

Figure 7 characteristic of callus chopping on characteristic of callus from mix flowers on MS medium supplemented with 2.0 mg/l BA and 1.5 mg/l 2,4-D after 4 weeks of culture (bars=0.5 cm)

a: 50 times b: 100 times c: 150 times d: 200 times

4. Effect of types of explants on somatic embryo (SE) formation

In this study, petal, single flower and mix flowers- derived calli were achieved on MS medium supplemented with 2.0 mg/l BA and 1.5 mg/l 2,4-D. The callus grew very fast on this culture medium. After culture for 12 weeks with subculture 4-week intervals, SE was developed from peripheral cells of callus in all

explants. The frequency of somatic embryogenesis and average number of SEs per callus increased when time of subculture increased. The callus derived from mix flower gave the highest percentage of SE formation at 39.84 and number of globular (Figure 8a), heart shaped (Figure 8b) and cotyledonary staged SEs (Figure 8c) per callus at 7.43, 4.52 and 3.25 SEs, respectively (Table 2), significantly different with another explants.

The induction of somatic embryogenesis in many plants, including Hevea, appears to be influenced by a number of variables, including developmental stages, explant types, plant growth regulators, basal media composition, light intensity, etc. In this work, BA and 2,4-D supplemented MS medium might generate SEs and encourage their development into the mature stage (cotyledonary SEs). Kongkeaw (2017) also observed that MS-based plant regeneration medium with BA and 2,4-D could promote plant regeneration through SEs from green budwood culture of *Hevea* but different concentrations were obtained. In case of green budwood culture, low concentration of BA at 0.5 mg/l and high concentration of 2,4-D at 2.0 mg/l required. However, plantlet regeneration in this study gave the lower rate of of *Hevea* which was similar to our result. The results gave higher rate than that obtained by Kongeaw (2017). This might be due to higher meristematic activity. Mix flowers explant contains many meristem cells which has higher meristematic activity than green budwood due to meristem cells were a group of cells that reside at the shoot and root tips or young explants. As undifferentiated (or slightly differentiated cells) they were considered as stem cells given that they were the origin of many of the cells that go on to rapidly differentiate/specialize and form (Doerner, 1999). Thus, it gave the best result in SE induction and plantlet regeneration. Additionally, a number of researches found that adding 0.06 mg/l NAA and 0.03 mg/l BA to MS medium improved SE induction and plant conversion rates in a variety of plant species, including mangosteen and pawa (*Garcinia speciosa* Wall) and somkhag (*G.*

atroviridis Griff.) (Te-chato, 1997). The development of SE directly from the explants was occasionally caused by the low amount of auxin and cytokinin in the SE induction media, suggesting that they did not develop through callus formation (Kouassi *et al*., 2008). But as a result of callus formation, our findings indicated that SEs were indirectly produced.

Table 2 Effect of types of explants on SE formation on MS medium supplemented with 2.0 mg/l BA and 1.5 mg/l 2,4-D subculture every 4 weeks for 12 weeks

Kinds of explants	SE formation (%)	No. of SEs/callus		
		Globular embryo	Heart shaped embryo	Cotyledonary embryo
lTCL	0	0	0	0
Petal	28.46^{c}	3.24^{b}	2.18^{b}	1.46^{b}
Single flower	34.66^{b}	3.48^{b}	1.86^{b}	1.25^{b}
Mix flowers	39.84^{a}	7.43^{a}	4.52^{a}	3.25^{a}
F-test	**	**	**	**
C.V. (%)	14.25	12.64	10.18	18.74

** = significantly different (P ≤ 0.01)

Means followed by the same letter within column are not significantly different according to DMRT

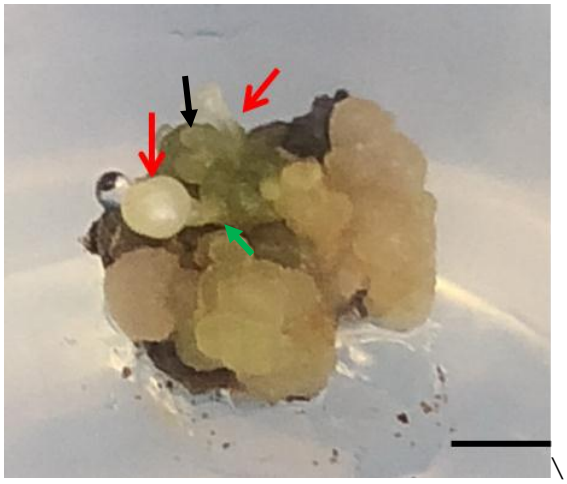

Figure 8 Different stages of SE formation (red arrows) from callus of mixed flowers on MS medium supplemented with 2 mg/l BA and 1.5 mg/l 2,4-D after 12 weeks of culture (subculture every 4 weeks) (bars= 0.2 cm)
a: Globular embryo (green arrow)
b: Heart-shaped embryo (black arrow)
c: Cotyledonary embryo (red arrows)

5. Effects of types of explants and GA_3 on development of SE

In this study, SEs at cotyledonary stage were obtained on MS medium supplemented with 2.0 mg/l BA, 1.5 mg/l 2,4-D and 0.25 mg/l GA_3 after culture for 4 weeks. The results revealed that SEs from mix flowers cultured on those PGRs containing medium gave the highest plant regeneration at 50% (Figure 9a), significantly different with other SEs from another explants. Plant regeneration obtained from single flower and petal was 37.5% and 12.5%, respectively (Table 3). Similar result was reported by Shamima *et al*. (2014) who observed that somatic embryos of *Wedelia calendulacea* Less. germinated into plantlets upon transfer to GA_3 containing MS medium. However, concentration of GA_3 used in the present study was two times lower than that used in *Wedelia calendulacea* Less. The reason might be due to different plant species cause the different response to the concentration

of GA_3. It is possible that high concentration of BA used in the present study might have synergistic effect to low concentration of GA_3. Like the report from Vengadesan and Paula (2009). For *Quercus rubra* L. somatic embryos, they discovered that the combination of BA and GA_3 favored simultaneous development of the shoot and root and produced a higher germination frequency. In the study, about 61% of the embryos germinated and developed normal shoots and roots on MS containing BA and GA_3. Kim *et al*. (2007) also achieved similar results when employed GA_3 in germination of somatic embryo *Podophyllum peltatum* L. However, some authors reported the reverse concentrations or proportions of both PGRs in germination of SEs. Palomo-Ríos *et al*. (2012) reported a significant improvement in the germination rate of genetically transformed avocado SEs when they were cultured in MS liquid medium with 0.1 mg/l BA and 10.0 mg/l GA_3 for 3 days. Xiao and Branchard (1993) applied high concentrations GA_3 (34.6 mg/l) for the initiation of embryogenic callus of *Spinacia oleracea*. Different plant species respond to different concentrations of GA_3 for elongation due to different concentrations of endogenous PGRs in those plants and explants. GA_3 have also been used for elongation of regenerated shoots (Lakshmi *et al*., 2013). The ability of GA_3 to complete embryo development may result from the activation of genes or the creation of novel gene products (Shamima *et al*., 2014). However, in this study, some SEs induced only shoot (Figure 9b). When effective roots fail to form, it may be due to meristem dysfunction or asynchronous development and a second rooting process is necessary to restore the entire plantlet, as has been observed in certain other species (Martin, 2004; Karami *et al*., 2006; Pacheco *et al*., 2007).

Table 3 Effects of types of explants and GA_3 on development of SEs on MS medium supplemented with 2.0 mg/l BA and 1.5 mg/l 2,4-D after 4 weeks of culture

Types of explants	GA_3 (mg/l)	Complete plantlet (%)	Only shoot (%)
Petal	0	0	0
	0.25	12.5[d]	12.5[b]
	0.50	12.5[d]	12.5[b]
	0.75	0	0
	1.00	0	0
Single flower	0	0	0
	0.25	37.5[b]	12.5[b]
	0.50	25.0[c]	0
	0.75	25.0[c]	0
	1.00	12.5	0
Mix flower	0	0	0
	0.25	50.0[a]	25.0[a]
	0.50	25.0[c]	0
	0.75	12.5[d]	12.5[b]
	1.00	12.5[d]	0
F-test		**	**
C. V. (%)		17.58	34.90

** = significantly different ($P \leq 0.01$)

Means followed by the same letter within column are not significantly different according to DMRT

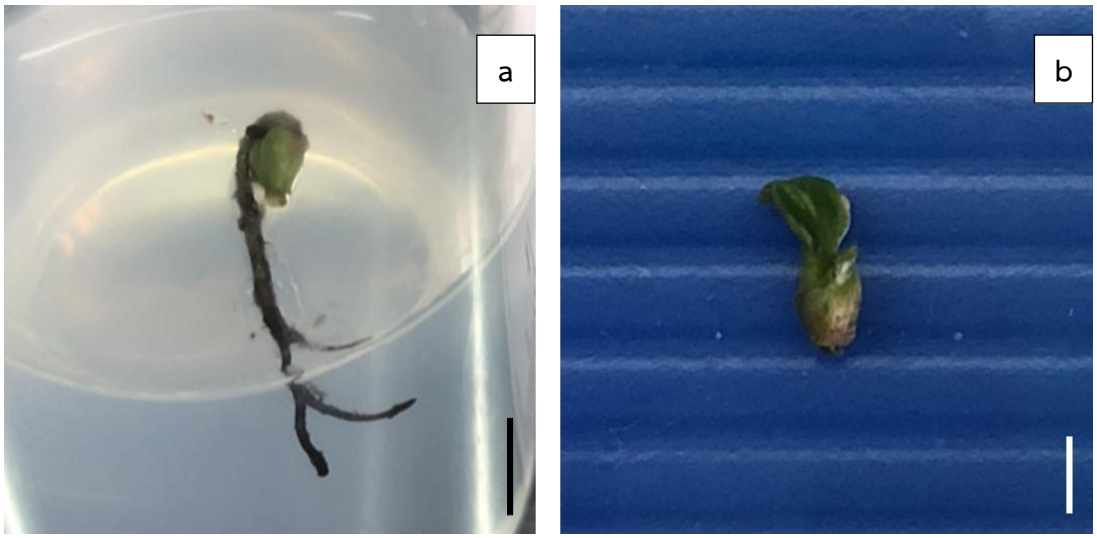

Figure 9 Plant regeneration from SE derived from mix flowers on MS medium supplemented with 2.0 mg/l BA, 1.5 mg/l 2,4-D and 0.25 mg/l GA_3 after 4 weeks of culture

a: Cotyledon with root (bar= 0.5 cm)

b: Shoot (bar= 0.2 cm)

6. Assessment genetic fidelity

The most important factor in plant propagation by tissue culture is to maintain genetic integrity with regard to the mother plants (MP). But it's well known that in vitro cultivation methods may bring about genetic variety, namely somaclonal variation (Jin *et al*., 2008). Somaclonal variation in tissue culture is a complex problem that needs several approaches to detect correctly (Leva *et al*., 2012). This is a concern in commercial micropropagation because it might have a detrimental impact on output and diminish the consistency of elite genotypes (Palombi *et al*., 2002). Recent research has shown that polyploidy, aneuploidy, chromosomal breakage, deletion, translocation, gene amplification, and mutations are prevalent genetic alterations in cell or tissue cultures. These changes are also manifested at the biochemical or molecular levels (Teixeira et al., 2007). In plant tissue culture and regenerants of various plants, somaclonal variation has been highlighted using various molecular analytical approaches. In order to explore genetic diversity in tissue culture-regenerated plants, RAPD and SSR are frequently utilized.

In the current work, POAD-01 and OPAD-10, two of the three RAPD-primers evaluated, were able to amplify and give monomorphic DNA patterns among in vitro rubber plantlets. Each primer had somewhere between 6 and 7 bands, with an average of 6.5 pieces per primer. Products that were amplified ranged in size from 300 to 1350 base pairs (Figure 10 and 11). 53 EIR clones of the rubber tree were subjected to genetic investigation by Nakkanong *et al*. (2008) using 8 RAPD primers (OPB-17, OPN-16, OPR-02, OPR-11, OPZ-04, OPAD-01, OPAD-10 and OPAD-12). One RAPD primer (OPAD-01) produced a 700-bp fragment that was exclusively found in the EIRpsuI clone, according to the data. According to Wattanasilakorn *et al*. (2015), the genetic linkages between the early-introduced clones were assessed using OPAD-01 and OPAD-10 primers, as well as RRIM 600, and DNA fragment polymorphism using RAPD primers. Base on the finding of Nakkanong *et al*. (2008) and Wattanasilakorn *et al*. (2015) RAPD primer OPAD-01 and OPAD-10 could be used to verify uniformity of rubber tree plantlets obtained from tissue culture technique. In previous study, Sirisom and Te-chato (2013b) used eight RAPD-primers including OPAD-01 and OPAD-10 to verify genetic stability of microcutting rubber tree. They also found that these two primers provided monomorphic patterns of DNA among *in vitro* rubber plantlets. The outcomes of this study's RAPD analysis were comparable to those of the SSR analysis. Each of the three SSR primers—hmac4, hmct1, and hmct5—could amplify and produce monomorphic DNA patterns. 1 to 10 pieces per primer produced a different amount of bands. Products that had been amplified varied in size from 200 to 300 bases pairs. The three primer pairings yielded a total of three SSR fragments (Figure 12-14). *In vitro* rubber plantlets produced from several explants did not exhibit any somaclonal variation, according to the results of the aforementioned two procedures. These SSR primers were reported to produce polymorphic DNA patterns among early induce clones of rubber tree collected from different areas in southern Thailand by Nakkanong *et al*. (2008) and used to screen rubber rootstock and genetic background by Wattanasilakorn *et al*. (2015). However, these three primers provided monomorphic DNA pattern in this present study. Hence, these primers could be used to verify genetic uniformity of *in vitro* rubber plantlets obtained from this

propagation like the assessment of somaclonal variation from *in vitro* plantlets derived from shoot tips or nodal by Sirisom and Te-chato (2013b).

An earlier work by Hua *et al*. (2010) demonstrated that in vitro cultivation of an anther from a rubber tree resulted in malformed embryos (CATAS 7-33-97 and CATAS 88-13 clones). Many factors involve in the freqeuency of somaclonal variation (Kaeppler *et al*., 2000). Among those, types of explants play an important role in the genetic stability of *in vitro* cultures (Jin *et al*., 2008). When regeneration is accomplished from various tissue sources, there may be differences in the frequency and character of somaclonal variation (Sahijram *et al.,* 2003). In this study, plantlets obtained from different explants (petal, single flower and mix flower) had the same genetic constituents as assessed by RAPD and SSR analysis. The results revealed that the analyzed of DNA from both protocols showed monomorphic patterns between mother plant and *in vitro* plantlets. Axillary buds and shoot tips are two examples of explants with preexisting meristems, but certain cases of differentiated tissues, such as roots, leaves, and stems, typically yield more variants (Duncan, 1997). Auxins, namely 2,4-D, are known to be necessary for somatic embryogenesis induction and embryo multiplication, both of which may be created through indirect somatic embryogenesis (Lloyd *et al*. 1980; Pasternak, 2002; Raghavan, 2004; Vondráková *et al*., 2011). For development and maturation steps it is necessary to decrease the concentration or remove this PGR from culture medium (Pasternak, 2002; Zavattieri *et al*., 2010; Garcia *et al*., 2019). According to the abnormality index, several researches claimed that BA was the most harmful cytokinin and that it might cause somaclonal variation in bananas (Bairu *et al*., 2008). It seems incredibly complicated how cytokinin affects embryo induction and development (Siragusa *et al*., 2007). However, in this present study, the concentration of auxin and cytokinin (1.5 mg/l 2,4-D and 2 mg/l BA) did not have an effect on somaclonal variation.

Table 4 DNA quantification analysis from leaf sample of mothenal se-derived plantlets from various explants (petal, single and mix flowers)

Treatments	Optical density at 260/280	Total DNA (ng/μl)
MP	1.92	2,248.3
S1	1.5	281.2
S2	2.0	284.4
S3	1.98	2,073.8
S4	2.04	289.1
S5	2.07	441.8
S6	2.05	461.6
S7	1.93	295.5
S8	2.11	263.9
S9	1.96	584.4

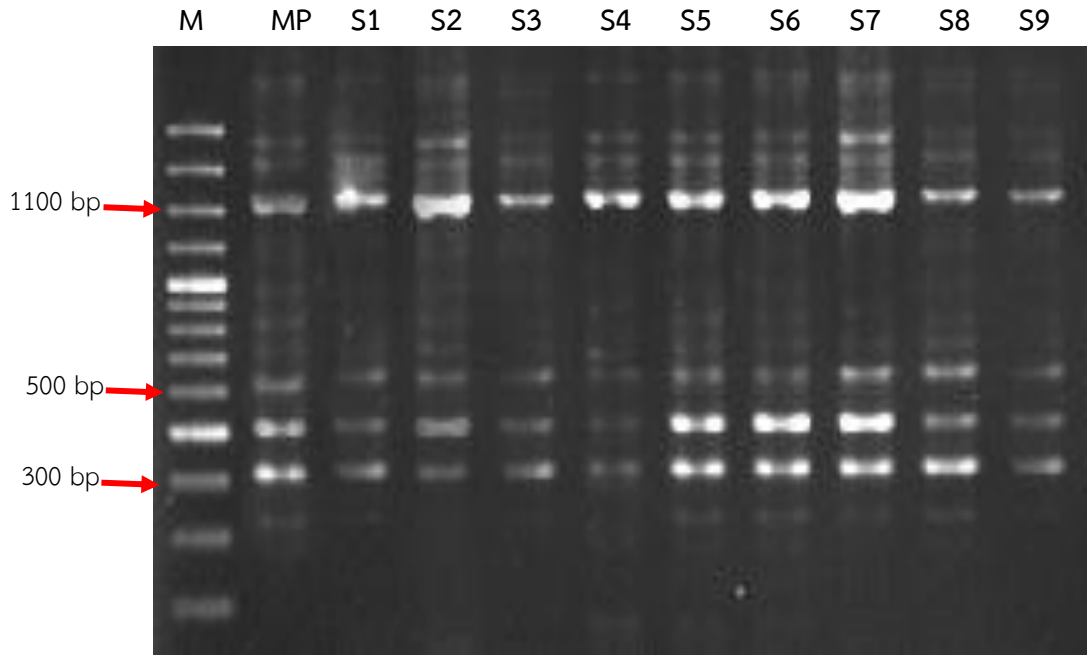

Figure 10 RAPD patterns of micropropagated plantlets compared to mother plant as amplified by primer OPAD-01

Lane M = 100 bp ladder, MP = mother plant, S1-S9 = DNA of *in vitro* young leaves samples

(S1-3 = petal, S4-6 = single flower and S7-9 = mix flower)

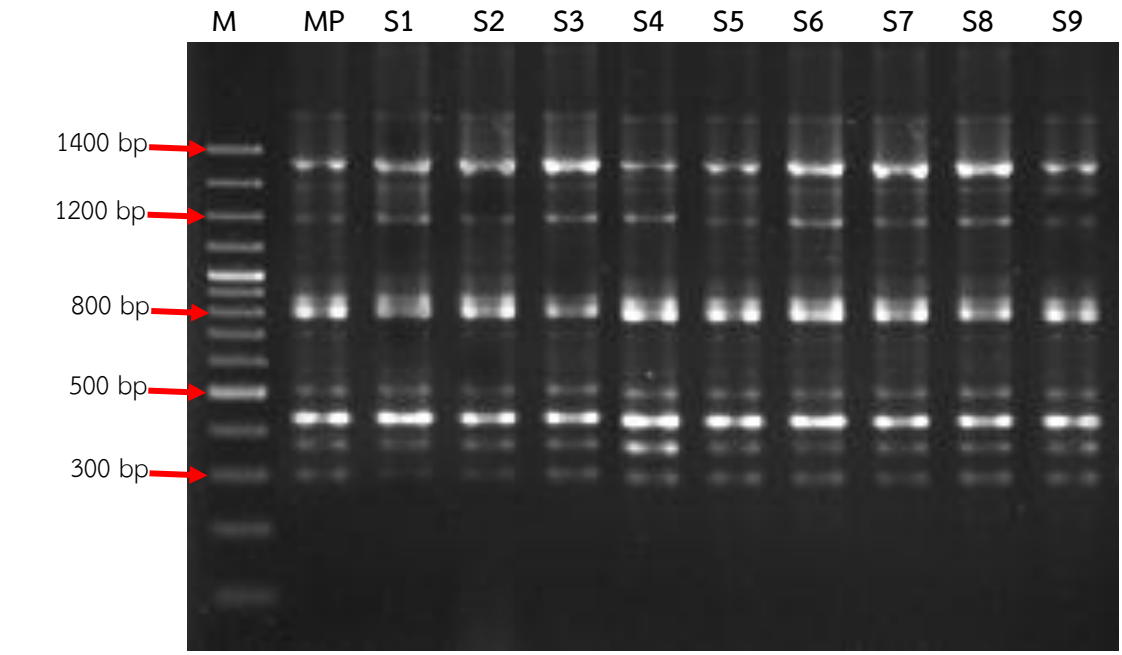

Figure 11 RAPD patterns of micropropagated plantlets compared to mother plant as amplified by primer OPAD10

Lane M = 100 bp ladder, MP = mother plant, S1-S9 = DNA of *in vitro* SE leaves samples

(S1-3 = petal, S4-6 = single flower and S7-9 = mix flower)

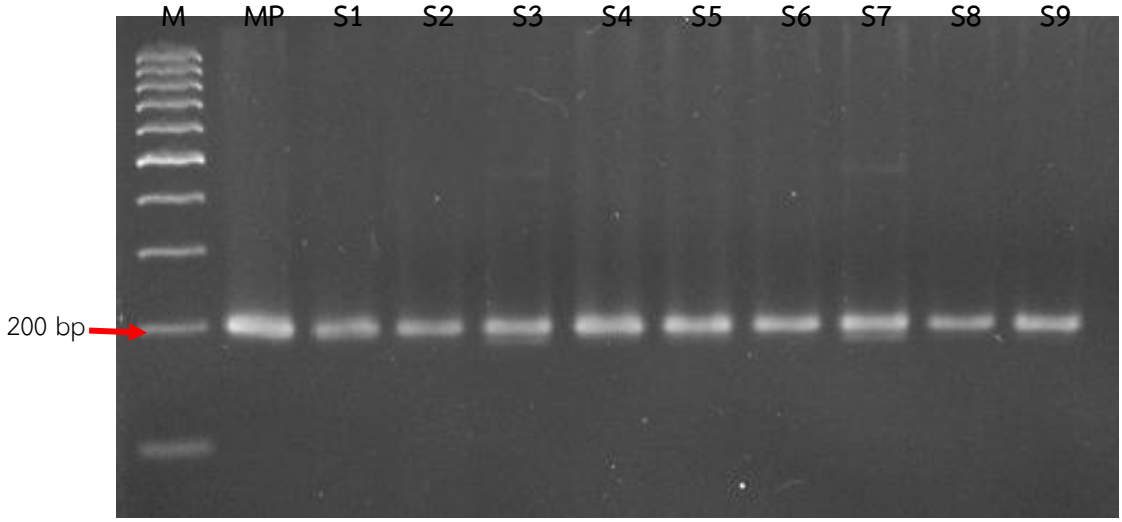

Figure 12 SSRs patterns of micropropagated plantlets compared to mother plant as amplified by primer *hmac4*

Lane M = 100 bp ladder, MP = mother plant, S1-S9 = DNA of *in vitro* young leaves samples.

(S1-3 = petal, S4-6 = single flower and S7-9 = mix flower

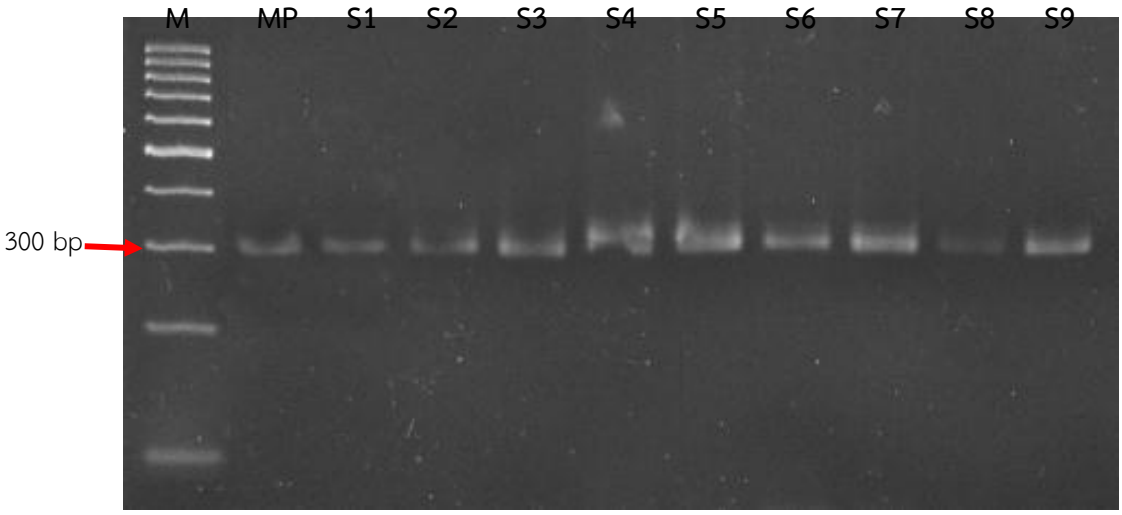

Figure 13 SSRs patterns of micropropagated plantlets compared to mother plant as amplified by primer *hmct1*

Lane M = 100 bp ladder, MP = mother plant, S1-S9 = DNA of *in vitro* young leaves samples.

(S1-3 = petal, S4-6 = single flower and S7-9 = mix flower)

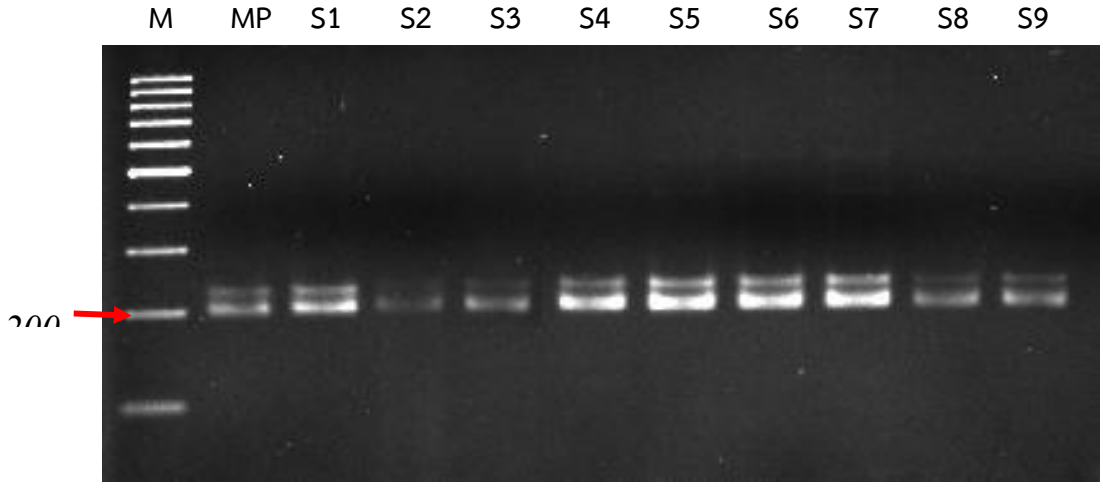

Figure 14 SSRs patterns of micropropagated plantlets compared to mother plant as amplified by primer *hmct5*

Lane M = 100 bp ladder, MP = mother plant, S1-S9 = DNA of *in vitro* young leaves samples.

CHAPTER 5
SUMMARY

NaOCl at 0.525% gave the best result in sterilization of all the explants (lTCl, petal, single flower and mix flowers) at 100%. All of explants cultured on MS medium supplemented with 2.0 mg/l BA and 1.5 mg/l 2,4-D gave the highest percentage of callus formation at 100% after culture for 4 weeks. Callus derived from mix flower chopped at number of 100 times gave the highest callus proliferation as determined by fresh weight at 392.05 mg after culture for 4 weeks. After 12 weeks of sub-cultured the callus derived from mix flowers gave the highest SE formation at 39.84% and number of cotyledonary embryo (CE) at 3.25 embryos /explant. GA_3 at 0.25 mg/l with the best concentration of BA and 2,4-D gave the best result in plant regeneration at 50% after culture for 4 weeks.

Assessment of somaclonal variation was carried out by RAPD and SSR markers. Plantlets obtained by this procedure had the same profiles of DNA among each other and mother plant as revealed by 2 RAPD primers; OPAD01 and OPAD10 and 3 SSR primers; *hmac4, hmct1 and hmct5* It is concluded that somaclones obtained from this protocol is uniform and have the same genetic constituents as mother plant.

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Appendix A

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

Component	Concentrations (mg/L)
Macro elements	
NH_4NO_3	1,650
KNO_3	1,900
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
pH 5.7	

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Lists of Publications and Proceedings

Publications

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