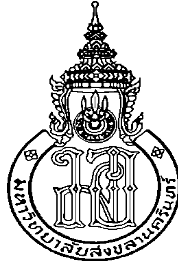

Proliferation of Embryogenic Callus Using Different Culture System, Direct Somatic Embryo Formation from Seedling Roots of Oil Palm and Assessment of Somaclonal Variation Using Simple Sequence Repeat (SSR) Technique

Stapaporn Kerdsuwan

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

Prince of Songkla University

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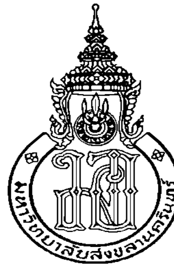

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Major Program	Plant Science

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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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(Miss Stapaporn Kerdsuwan)

Candidate

ชื่อวิทยานิพนธ์ การเพิ่มปริมาณเอ็มบริโอเจนิคแคลลัสโดยใช้ระบบการเลี้ยงที่ต่างกัน การสร้างต้นอ่อนโดยตรงจากต้นกล้าปาล์มน้ำมันและการประเมินความแปรปรวนทางพันธุกรรมโดยเทคนิคเอสเอสอาร์
ผู้เขียน นางสาวศตปพร เกิดสุวรรณ
สาขาวิชา พืชศาสตร์
ปีการศึกษา 2558

บทคัดย่อ

ศึกษาผลของ dicamba ต่อการเพิ่มปริมาณเอ็มบริโอเจนิคแคลลัส และการเกิดโซมาติกเอ็มบริโอจากเอ็มบริโอเจนิคแคลลัสปาล์มน้ำมันที่เพาะเลี้ยงในอาหารสูตร OPCM (Oil Palm Culture Medium) เติม dicamba ที่ความเข้มข้น ต่าง ๆ โดยย้ายเลี้ยงทุก ๆ เดือน เป็นเวลา 3 เดือน ส่วนการชักนำโซมาติกเอ็มบริโอโดยตรงจากรากต้นปาล์มน้ำมันอายุ 3 เดือน ที่เพาะเลี้ยงบนอาหารสูตร OPCM MS (Murashige and Skoog) หรือ WPM (Woody Plant Medium) เติม NAA (α- naphthalene acetic acid) dicamba หรือ 2,4-D (2,4-Dichlorophenoxyacetic acid) เข้มข้น 0-1 มิลลิกรัมต่อลิตร เติมน้ำตาลซูโครส 3 เปอร์เซ็นต์ กรดแอสคอร์บิคเข้มข้น 200 มิลลิกรัมต่อลิตร และผงวุ้น 0.75 เปอร์เซ็นต์ วางเลี้ยงในสภาพที่มีแสง 15 ไมโครโมลต่อตารางเมตรต่อวินาที โดยให้แสง 14 ชั่วโมงต่อวัน อุณหภูมิ 27±2 องศาเซลเซียส เป็นเวลา 2 เดือน นอกจากนี้ยังประเมินประสิทธิภาพการเพิ่มปริมาณเอ็มบริโอเจนิคแคลลัสของปาล์มน้ำมันโดยเปรียบเทียบวิธีการเพาะเลี้ยงในอาหารแข็ง อาหารเหลว และในระบบจุ่มแช่ชั่วคราว ในอาหารสูตร MS เติม dicamba เข้มข้น 0.1 มิลลิกรัมต่อลิตร กรดแอสคอร์บิคเข้มข้น 200 มิลลิกรัมต่อลิตร และน้ำตาลซูโครส 3-3.75 เปอร์เซ็นต์ โดยให้อาหารครั้งละ 3 นาที ทุก ๆ 4, 8, 12 และ 16 ชั่วโมง ปริมาณอาหารที่ใช้ 50, 100, 150, 200 และ 250 มิลลิลิตร เป็นเวลา 1 เดือน จากการศึกษาผลของ dicamba พบว่า อาหารสูตร OPCM เติม dicamba เข้มข้น 0.1 มิลลิกรัมต่อลิตร ให้น้ำหนักสดของเอ็มบริโอเจนิคแคลลัสสูงสุด 0.33 กรัม และให้โซมาติกเอ็มบริโอเฉลี่ยสูงสุด 30 เปอร์เซ็นต์ เมื่อนำโซมาติกเอ็มบริโอ มาเพาะลี้ยงบนอาหารสูตร MS ที่ปราศจากสารควบคุมการเจริญเติบโต ส่งเสริมการสร้างยอดสูงสุด 10.4 ยอดต่อชิ้นส่วน จำนวนรากเฉลี่ยสูงสุด 8.2 รากต่อชิ้นส่วน และให้การพัฒนาเป็นต้นที่สมบูรณ์เฉลี่ยสูงสุด 7.2 ต้นต่อชิ้นส่วน ส่วนการศึกษาการชักนำโซมาติกเอ็มบริโอโดยตรงจากต้นปาล์มน้ำมัน เมื่อเพาะเลี้ยงในอาหารสูตร OPCM เติม NAA เข้มข้น 0.5 มิลลิกรัมต่อลิตร พบว่า มีจำนวนโซมาติกเอ็มบริโอมากที่สุด 1.2 โซมาติกเอ็มบริโอต่อราก

และอัตราการเกิดโซมาติกเอ็มบริโอสูงสุด 80 เปอร์เซ็นต์ จุดกำเนิดของโซมาติกเอ็มบริโอดังกล่าวมาจากเซลล์ชั้นเอพิเดอร์มิส (epidermis) และพาเรนไคมา (parenchyma) สำหรับการศึกษาการเพิ่มปริมาณเอ็มบริโอเจนิคแคลลัส เป็นไปได้ดีในอาหารเหลวสูตร MS เติม dicamba เข้มข้น 0.1 มิลลิกรัมต่อลิตร น้ำตาลซูโครส 3.75 เปอร์เซ็นต์ ระบบการจุ่มแช่ชั่วคราว โดยให้อาหารครั้งละ 3 นาที ทุก ๆ 12 ชั่วโมง ปริมาณอาหารที่ใช้ 150 มิลลิลิตร ให้อัตราการเจริญเติบโตของเอ็มบริโอเจนิคแคลลัสสูงสุดที่ 11.19 เท่า แตกต่างทางสถิติอย่างมีนัยสำคัญกับระบบการเพาะเลี้ยงแบบอื่น ๆ เมื่อตรวจสอบความแปรปรวนทางพันธุกรรมของเอ็มบริโอเจนิคแคลลัสที่ได้จากระบบจุ่มแช่ชั่วคราวโดยใช้เทคนิคเอสเอสอาร์ จำนวน 9 ไพรเมอร์ พบว่า ทุกไพรเมอร์ให้แถบดีเอ็นเอได้ชัดเจน และไม่พบความแปรปรวนทางพันธุกรรม

Thesis Title	Proliferation of Embryogenic Callus Using Different Culture System, Direct Somatic Embryo Formation from Seedling Roots of Oil Palm and Assessment of Somaclonal Variation Using Simple Sequence Repeat (SSR) Technique
Author	Miss Stapaporn Kerdsuwan
Major Program	Plant Science
Academic Year	2015

ABSTRACT

The effects of dicamba on multiplication of embryogenic callus and somatic embryo on OPCM (Oil Palm Culture Medium) supplemented with different concentrations of dicamba were evaluated. The calli were subcultured to fresh medium with the same component at monthly intervals for 3 months. Besides direct induction of somatic embryos (SEs) from roots, 3-month-old oil palm plantlets regenerated from immature oil palm fruit (3 months after pollination: 3 MAP) were studied. The plants were cultured on OPCM, MS (Murashige and Skoog) or WPM (Woody Plant Medium) medium supplemented with 0-1 mg/L NAA (α-naphthalene acetic acid), dicamba or 2,4-D (2,4-Dichlorophenoxyacetic acid), 3% sucrose and 200 mg/L ascorbic acid and maintained under light intensity of 15 µmol/m2/s, 14 h photoperiod, 27±2 °C for 2 months. Furthermore, proliferation of embryogenic callus (EC) of oil palm using solid medium, liquid medium and temporary immersion system (TIS). For TIS, the EC was immersed in MS medium with 0.1 mg/L dicamba and different concentrations of sucrose (3-4%), immersed in 50-250 mL of volumes of medium for 3 min every 4, 8, 12 and 16 h. The results showed that the maximum fresh weight of EC at 0.33 g and the highest frequency of somatic embryo (SE) formation at 30% was obtained on OPCM containing 0.1 mg/L dicamba. Upon transferring SE to hormone-free MS medium, the highest number of shoots at 10.4 shoots/culture, number of roots at 8.20 roots/culture and complete plantlets at 7.20 plantlets/culture were obtained.

The highest number of direct SEs at 1.2 SEs/root and percentage of direct SE formation was recorded to be 80. Histological studies of direct SE formation from roots revealed that SEs were initiated from epidermal cells and a few parenchymatous cells. TIS was found to be suitable for EC proliferation. The EC cultured in MS medium supplemented with 0.1 mg/L dicamba, 200 mg/L ascorbic acid and 3.75% sucrose at a temporary immersion for 3 min every 8 h, with a 150 mL volume of medium, resulted in the highest growth ratio of EC at 11.19 folds, significantly different from other treatments. Verification of somaclonal variation in EC raised on TIS system by SSR techniques using 9 primers of SSR gave clear DNA patterns with monomorphism bands without somaclonal variation of EC.

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Stapaporn Kerdsuwan

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LIST OF ABBREVIATIONS AND SYMBOLS

ANOVA	=	Analysis of variance
APCS	=	Automated plant culture system
Avg	=	Average
B	=	Boron
BA	=	6-benzyladenine
BIB®	=	Bioreactor of immersion by bubble
BioMINT™	=	Modular temporary immersion bioreactor
BIT®	=	Twin flasks system
BL	=	Blade medium
bp	=	Base pair
BTBB	=	Balloon type bubble
Ca	=	Calcium
Cl	=	Chlorine
CRD	=	Completely random design
CTAB	=	Cetyltrimethyl ammonium bromide
Cu	=	Copper
2,4-D	=	2,4-Dichlorophenoxyacetic acid
Dicamba	=	3,6-dichloro-o-anisic-acid
DMRT	=	Duncan’s multiple range test
DNA	=	Deoxyribonucleic acid
dNTP	=	Deoxynucleotide triphosphate
EC	=	Embryogenic callus
EDTA	=	Ethylenediaminetetraacetic acid
Fe	=	Iron
gFW	=	Gram fresh weight
HE	=	Haustorium
HCl	=	Hydrochloric acid
IZEs	=	Immature zygotic embryos

LIST OF ABBREVIATIONS AND SYMBOLS (continued)

K	=	Potassium
KOH	=	Potassium hydroxide
M	=	Molar
MAP	=	Months after pollination
MAS	=	Marker assisted selection
mL	=	Milliliter
mm	=	Millimeter
mM	=	Millimolar
Mg	=	Magnesium
mg/L	=	Milligram per liter
Mn	=	Manganese
Mo	=	Molybdenum
MS	=	Murashige and Skoog (medium)
MZEs	=	Mature zygotic embryos
N	=	Nitrogen
NAA	=	α- naphthalene acetic acid
NaCl	=	Sodium chloride
Na_2EDTA	=	Disodium ethylenediaminetetraacetate
Ni	=	Nickel
ns	=	Not significant difference
OPCM	=	Oil palm culture medium
P	=	Phosphorus
PCR	=	Polymerase chain reaction
PEMs	=	Proembryogenic masses
PGRs	=	Plant growth regulators
PPM™	=	Plant cell technology
PVP	=	Polyvinyl pyrrolidone
RAPD	=	Random amplified polymorphic DNA

LIST OF ABBREVIATIONS AND SYMBOLS (continued)

RITA®	=	Automated temporary immersion
rpm	=	Round per minute
S	=	Sulfur
SEs	=	Somatic embryos
SSEs	=	Secondary somatic embryos
SSR	=	Simple sequence repeats
TE	=	Tris Ethylenediaminetetraacetic acid
TAE	=	Tris-acetic acid-disodium ethylenediaminetetraacetic acetate
TBE	=	Tris-boric acid-disodium ethylenediaminetetraacetic acetate
TIB	=	Temporary immersion bioreactor
TIS	=	Temporary immersion system
Tris	=	Tris (hydroxymethyl) aminomethane
v/v	=	Volume per volume
WPM	=	Woody plant medium
w/v	=	Weight per volume
µl	=	Microlitter
$\mu mol\ m^{-2}\ s^{-1}$	=	Micromole per square meter per second
µM	=	Micromolar
Zn	=	Zinc

LIST OF PAPERS AND PROCEEDINGS

1. Kerdsuwan, S. and Te-chato, S. 2014. Effect of dicamba on induction of somatic embryo and plant regeneration of oil palm (*Elaeis guineensis*). Songklanakarin Journal of Plant Science 1: 2-9.

2. Kerdsuwan, S. and Te-chato, S. 2016. Direct somatic embryo formation from roots of vitro-seedlings of oil palm (*Elaeis guineensis* Jacq.). Walailak Journal of Science and Technology 13: 45-53.

3. Kerdsuwan, S. and Te-chato, S. 2016. Proliferation of embryogenic callus of oil palm (*Elaeis guineensis* Jacq.) using different culture systems and genetic instability assay by Simple Sequence Repeat (SSR) technique (in press).

General Introduction

1.1 Background

Oil palm (*Elaeis guineensis* Jacq.) is a diploid monocotyledon belonging to the family Areaceae. It is an economically important product, as it is the source of palm oil, the most traded vegetable oil in the international market and it is increasing used in the food industry (Corley and Tinker, 2003). Given the increasing economic value of oil palm, micropropagation of this tropical crop has been studied to develop oil palm propagation with a view to the commercial distribution of elite clones for increasing palm oil production (de Touchet *et al*., 1991). Clonal propagation of oil palm has been studied for many years as a potential way to develop high-yielding collections while circumventing the long generation time required with traditional breeding techniques. In 1991, the first regenerable oil palm embryogenic cell suspension cultures were obtained from fruits used to generate friable embryogenic tissue and to establish embryogenic suspension (Teixeira *et al*., 1995). Although these two protocols were successful for establish single somatic embryos from embryogenic suspension cultures, the regeneration rate into plantlet was poor. This issue was overcome by Aberlenc-Bertossi *et al*. (1999), who developed a protocol for improving the quality of shoot formation. The first result obtained from suspension culture derived clones are encouraging and field trails are under way. Most of these protocols used solidified medium, and its efficiency is rather low considering that the multiplication rate for most protocols is more than 60 days and liquidified medium with agitation gave better results during the multiplication stage (Lee-Espinosa *et al*., 2008). Temporary immersion system (TIS), among other advantages, allows for the semi-automation of the culture process, facilitates medium renewal, and also combines aeration and explant immersion with programmed duration and frequency of immersion, thus reducing hyperhydricity of the plant and increasing survival rate (Levin and Tanny, 2004). The TIS has been used in the micropropagation of several species including *Malus* sp. (Zhu *et al*., 2005), *Eucalyptus* spp.

(McAlister *et al*., 2005), *Saccharum* spp. (Mordocco *et al*., 2009) and *Quercus robur* (Mallon *et al*., 2012).

Recently, molecular marker techniques based on the polymerase chain reaction (PCR) has been one of the most commonly used on the characterization of genetic variability, genotype identification, genetic instability analyses, selection and breeding purposes (Tirgey and Tufo, 1993). Among those techniques simple sequence repeat (SSR) was proved to be very commonly used in identification F_1-hybrid of oil palm (Thawaro and Te-chato, 2009), verification of uniformity of oil palm plantlets from tissue culture (Sanputawong, 2010) and population genetic studies in plants such as rice (Cho *et al*., 2000), Wheat (Eujayl *et al*., 2001), and potato (Provan *et al*., 1996). They have also been widely used in genetic mapping studies in crops like *Eucalyptus* species (Brondani *et al*., 1998), barley (Ramsay *et al*., 2000) and more recently in oil palm (Billotte *et al*., 2005). These studies have confirmed the importance of SSR as a source of markers for plant genetics.

1.2 Origin of the oil palm

The oil palm has been postulated to originate in Gondwanaland which disappeared when the American and African continents drifted apart in prehistoric times (Zeven, 1965) giving rise to the evolution of African oil palm and American oil palm (*E. oleifera* or *E. melanoccoca*). The oil palm gives the highest oil yield per hectare in comparison with all oil crops at present. The fruit of the palm has a central hard-shelled nut surrounded by an outer mesocarp which contains the normal palm oil of commerces. The nut contains the palm kernel, from which a different type of oil, palm kernel oil, is extracted by pressing, leaving a proteinaceous residue that is a valuable animal seed, palm kernel cake. The high and increasing yields of the oil palm have led to a rapidly expanding world industry, now based in the tropical areas of Asia, Africa and America. Its origin is believed to

have been in Africa, but the most productive parts of the industry at present are in Malaysia and Indonesia, which provide most of the oil entering international trade. In a cross-pollinating crop such as the oil palm, progenies will be genetically heterogeneous if reproduction is by way of seed, and the potential yield for each plant will be different. If it were possible to produce genetically uniform plants, and if these were of superior genotypes, considerable yield increases would be possible.

In many perennial crops genetically uniform material can be produced by vegetative propagation, using suckers, cuttings or grafts, where the genetic constitution of all 'offspring' is identical with that of the original material. All individual plants with the same genetic composition, produced from the same original material in such ways, are collectively called a clone. The members of a clone are known individually as ramets. With such techniques selected genotypes can be readily reproduced for commercial use. The greatest benefit of clonal propagation will be the yield increases possible; the resulting uniformity might also simplify management of harvesting and other aspects, although it may also have disadvantages where characteristics such as disease susceptibility are involved. The oil palm only has a single growing point, and does not produce suckers like some other palm species, so clones cannot be produced by the common techniques referred to above. However, it is possible to produce clones by tissue culture, in which small pieces of tissue explants are grown on special nutrient solutions. The plant from which the tissue is taken is known as an ortet. The growing tissue may form callus a mass of cells without differentiation, and this may be treated to produce embryonic tissue, that slowly grows into shoots. These shoots can be rooted and planted in a nursery, and thereafter handled in much the same way as ordinary seedlings. The tissue culture techniques are difficult and laborious, and the underlying biology is not properly understood, so that finding successful methods is very slow (Corley and Tinker, 2003).

1.3 Oil palm tissue culture

Attempts to propagate oil palm by tissue culture started in the 1960s, and by the mid-1970s success had been achieved. The early work was reviewed by Jones (1995). The first clonal palms were planted in the field in Malaysia in 1977 and the first replicated trials in 1978. Following this success, there was a rapid expansion of effort, and by the mid-1980s, there were at least ten laboratories in Malaysia doing oil palm tissue culture (Wooi, 1990) and several others elsewhere (Le Guen *et al*., 1991). The discovery of abnormal flowering and severe bunch failure caused a major setback, just as commercial exploitation was about to begin. Since the 1980s, there has been a great deal of research on the abnormal flowering problem, and by the late 1990s there was cautious expansion into commercial planting of clones. It has been known that the potential for multiplication and regeneration of oil palm tissue culture depends on a number of factors, such as the genotypes (Sanputawong and Te-chato, 2008), type and physiological of explant (Chehmalee and Te-chato, 2007), the organic components and PGRs in culture media (Thawaro and Te-chato, 2007).

1.3.1 Tissue sampling and callus formation

The tissue explants most commonly used are immature leaf or inflorescence, or the tips of tertiary roots. Roots are always heavily contaminated with soil fungi and bacteria, and need vigorous disinfection. Wooi *et al*. (1981) recommended 0.1% mercuric chloride followed by 10% sodium hypochlorite. There is also a danger of taking, in error, roots from an adjoining palm (Mayes *et al*., 1996). To avoid this problem, soil can be mounded up around the palm base and roots growing into it can be sampled, but even then care must be taken to exclude roots from neighboring palms growing into the mound from below, and roots of seedlings that may have germinated in leaf axils above the mound. Using leaf explants avoids any risk of error, and young leaf tissue does not require severe

disinfection, being completely enclosed by the bases of older leaves. The tissue must be immature, though; the bases of unopened leaves are used (Noiret *et al*., 1985) and excision causes a severe check to the palm's growth, with a risk of death. Explants cannot be done more frequently than every second or third year, and palms in use for a breeding program cannot be sampled. Wooi *et al.* (1981) found no differences in frequency of callusing between unopened inner most leaf number 1 to 7. Young inflorescence tissues from the axil of about leaf 10 give good results, and can be excised without damaging the palm too seriously (Teixeira *et al*., 1994), but general experience appears to be that callus initiation and embryogenesis occur sooner with leaf explants than with inflorescence (Eeuwens, 2002). Wooi (1981) found that there was no difference in callus frequency from root explants between palms aged 9–10, 14–16 and 22–23 years, whereas Paranjothy *et al.* (1990) found that young palms (3 years old) gave significantly higher callus frequencies than 10-year-old palms, from both leaf and root explants. However, the need for recording over several years to identify the best individuals means that sampling cannot usefully be done from 3-year-old palms. An auxin, usually 2,4-dichlorophenoxyacetic acid (2,4-D) or naphthalene acetic acid (NAA), is essential for callus initiation, but cytokinins are inhibitory (Paranjothy *et al*., 1990). Callus may be initiated within 2 months of putting explants into culture. Most callus grows slowly, forming a compact, nodular mass, but a fast-growing callus may develop spontaneously (Smith and Thomas, 1973) and can be induced on suitable media (Duval *et al*., 1995). Embryogenesis occurs readily on fast growing callus, but it appears that the embryoids are more likely to suffer from the flowering abnormality, so slow growing, nodular callus is preferred (Duval *et al*., 1988). Using leaf explants, all palms sampled give callus, with the proportion of mature palm explants giving callus ranging from 7 to 60% (Wong *et al*., 1999b).

1.3.2 Culture media composition

Plant tissues and organ are grown *in vitro* on artificial media, which supply the nutrients necessary for growth and development. The success of plant tissue culture as a means of plant propagation is greatly influenced by the nature of the culture medium used. Generally, the composition of culture media is consisted of; macronutrients, which provided the six major elements: nitrogen (N), phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg) and sulfur (S). This required for plant cell or tissue growth. The optimum concentration of each nutrient for achieving maximum growth rates varies considerably among species and micronutrients, which the essential for plant cell and tissue growth include iron (Fe), nickel (Ni), chlorine (Cl), manganese (Mn), zinc (Zn), boron (B), copper (Cu) and molybdenum (Mo). Culture medium was developed for optimal growth and the development of plant involved a large number of does-response curves for the various essential minerals. Medium salt composition has also been shown to affect the formation and further development of somatic embryos. Thus, culture medium plays significant role on plant development. In oil palm tissue culture, mostly researchers reported the use of basal MS medium (Murashige and Skoog, 1962) or modification of this medium, half strength MS medium (½MS), Y_3 medium (Eeuwens, 1978), Blade medium (BL), woody plant medium (WPM) (Lloyd and McCown, 1980) and oil palm culture media (OPCM) (Heedchim, 2514) as callus induction and proliferation and germination medium. Patcharapisutsin (1990) reported that mature zygotic embryos (MZEs) cultured on ½ MS medium supplemented with 0.05% activated charcoal gave seedling formation at 90% higher than that of full strength MS medium. Chourykaew (1990) reported that Y_3 medium supplemented 0.05% activated charcoal resulted in the best seedling formation in comparison with another culture medium. Srisawat and Kanchanapoom (2005) reported that MZEs cultured on PGR-free MS medium gave the highest percentage of germination than PGR-free Y_3 medium. Similar result with Chehmalee and Te-chato (2007) also found that MZEs of all crosses cultured on PGR-free

MS medium gave the highest percentage of germination at 16.67 when compared with other culture media. Thus, MS medium is the standard media widely used for culture tissues and has the sufficient nutrient requirement of oil palm germination. However, not only culture medium plays role on proliferation and germination, but also genotypes and PGRs (plant growth regulators) influence on available for proliferation and germination.

1.3.3 Plant growth regulators (PGRs)

Types and concentrations of PGRs in the culture media can greatly affect somatic embryo or somatic embryogenesis induction of oil palm tissue culture. Regeneration of culture cells is essential for crop improvement through biotechnology. Among the different auxin 2,4-D, a synthetic auxin, is the most widely used for oil palm callus induction at a concentration of 1-100 mg/L. A much of lower concentration of 2,4-D was applied as the sole sources of plant growth regulator in the regeneration medium to induce regeneration of cultured oil palm cells. Dicamba (3,6-dichloro-o-anisic-acid), another plant growth regulator and used as selective systemic herbicide against annual, perennial broad-leaved weeds and bush species (Pena-Ramirez *et al*., 2011) is also effective on induce callus formation. Dicamba has been reported to be an effective auxin for increasing a large number of somatic embryos (Te-chato *et al*., 2003a). NAA has also been reported to induce callus formation but high concentration at 40 mg/L was required (Te-chato, 1998). Unfortunately, development of somatic embryos through embryogenesis was not reported. The main effect of NAA was reported to induce the formation of a primary ortho-gravitropic root comparable of the developed during *in vitro* germination of oil palm seeds (Jourdand and Rey, 1997). The used of PGRs in oil palm is generally depending on the types of explants and the genetic origin of the material. Dicamba has been reported to be an effective auxin for increasing a large number of somatic embryos (SEs) (Te-chato *et al*., 2003b). Regeneration of oil palm through secondary somatic embryos (SSEs) has also been reported using polyamines (Rajesh *et al*., 2003). Te-chato *et al*. (2008) reported that the

combination of 0.1 mg/L KN with 0.1 mg/L dicamba gave small aggregates composed of isodiametric cells with dense cytoplasm led to establishment of fine suspensions in short period of time.

1.3.4 Embryogenesis and embryoid proliferation

It is usually easy to distinguish between embryogenic and non-embryogenic callus based on morphology and color. Embryogenic callus is composed of proembryogenic masses (PEMs). At present it is not known if the first formed PEM actually is an embryo which deviates from the normal embryonic development in response to PGRs and proliferates. Owing to the difficulty in strictly separating direct and indirect somatic embryogenesis it would be classified as indirect embryogenesis, where embryogenic callus is formed first. To execute this pathway efficiently, a number of critical physical and chemical treatments should be applied with proper timing. Although great progress has been made in the developments of these treatments and understanding of their mechanism of action, it has also been revealed that some maturation treatments, coinciding with increase yield of somatic embryo, may cause adverse effects on embryo quality, thereby impairing germination and *ex vitro* growth of somatic embryo plants. Consequently, *ex vitro* growth of somatic embryo plants is under a cumulative influence of the treatments provides during the *in vitro* phase. Plant regeneration via somatic embryogenesis includes five steps. The first step is initiation of embryo genetic cultures by culturing the primary explant on medium supplemented with PGRs, mainly auxin but often also cytokinin. The second step is proliferation of embryogenic cultures on solidified or in liquidified medium supplemented with PGRs, in a similar fashion to initiation. The third step is pre-maturation of somatic embryos in medium lacking PGRs, this inhibits proliferation and stimulates somatic embryo formation and early development. The fourth step is maturation of somatic embryos by culturing on medium supplemented with ABA or having reduced osmotic potential. The finally step is regeneration of plants on medium lacking PGRs (Soh *et al*., 2001).

A special process of direct somatic embryogenesis, is the process which usually is classified as secondary embryogenesis. Secondary embryogenesis is termed continuous, recurrent or accessory, when the first formed somatic embryo fails to develop into a plant but instead gives rise to successive cycle of embryos, secondary or tertiary. Secondary embryos develop directly from epidermal and sub-epidermal cells of the cotyledons or hypocotyls (Thomas *et al*., 1976). In some case the formation of secondary embryos is of significance importance for increasing the yield of plants regenerated. If directly formed somatic embryos are converted into plants, no further multiplication is possible. However, it is often difficult to stop the process and consequently no, or only a few, normal plants can be regenerated. The main problem is that according to order hypotheses direct embryogenesis should take place from embryogenic pre-determined cells. In contrast, indirect somatic embryogenesis should take place from undetermined cells and an undifferentiated callus should first be formed. However, in reality the callus formed is either embryogenic callus.

The multiplication necessary for a propagation system is achieved by embryoid proliferation. This involves subdividing the cluster of embryoids from a well developed old culture and inoculating several tubes of fresh proliferation medium, while transferring any shoots to rooting medium. The proliferation medium may be hormone free (Rival, 2000) or with auxin only, or auxin and cytokinin (Paranjothy *et al*., 1995). Only about 50% of embryoid 'lines' could proliferate while the remaining ones could not (Wong *et al*., 1999; Rival, 2000). The reasons for the lack of proliferation with other lines are unknown. An embryoid line consists of all the embryoids descended by proliferation from a single embryogenic event in a callus culture. In most laboratories, the number of subcultures per embryoid line is deliberately restricted, because of the possibility that the flowering abnormality may be related to time in culture. If this is done, then for large-scale propagation several lines per palm are needed, but Soh *et al*. (2001) reported that less than

20% of palms sampled gave more than ten proliferating embryoid lines. It is clearly seen that this situation must depend on the number of callus cultures established. Rival (2000) described this as the main stumbling block for large-scale clonal propagation. Wong *et al*. (1997) showed that proliferation was encouraged by shorter intervals between transfers to fresh culture medium, while Jones (1995) noted that short transfer intervals tended to increase the frequency of abnormal flowering.

1.3.5 Conditioning factors regulating somatic embryogenesis

The mechanism that controls cell differentiation in plant somatic embryos is far from clear, although there is evidence that soluble signal molecules are involved. It has long been observed that conditioned medium from embryogenic cultures could promote embryogenesis such as conditioned growth medium from highly somatic cultures can induce embryogenesis in non-embryogenic cultures (Hari, 1980). Growth medium pre-conditioned by a high density suspension culture can also induce embryogenesis in cells cultured at a low cell density. This ability of conditioned medium to sustain or stimulate somatic embryogenesis implies that secreted soluble signal molecules are important.

Based on the knowledge gained from studying the developmental pathway of somatic embryogenesis it is possible to construct a model of the process. The model has to be specific for each system. Below we give an example of a model for somatic embryogenesis in *Picea abies* involves two broad phases, which in turn are divided into more specific developmental stages. The first phase is represented by proliferating PGMs, cell aggregates which can process through a series of three characteristic stages distinguished by cellular organization and cell number (Stages PEM I,II and III), but can never develop directly into a real embryo. The second phase encompasses development of somatic embryos. The latter arise *de novo* from PEM III, and then process through the same, stereotyped sequence of stages as described for zygotic embryogeny of *Pinaceae* (Singh,

1978). Auxin and cytokinin are necessary during the first phase to maintain PEM proliferation, whereas embryo formation from PEM III is triggered by lacking of PGRs. Once early somatic embryos have formed, their further development to mature forms requires ABA. Each developmental stage within this fate map should be further characterized by a set of adequate molecular markers (Filonovo *et al*., 2000).

1.3.6 Direct regeneration

When relative large pieces of intact plants are transferred to nutrient media, new shoots, roots, somatic embryos and even flower initials are often formed without the intermediate growth of callus tissue. Small explants show organogenesis only rarely, although some exceptions have been reported. The part of the original plant from which the explant is taken is important in influencing its morphogenetic potential. Many species give rise to adventitious shoot buds on explants taken from a variety of organs including those derived from petioles, stems, roots, leaves and cotyledons. By contrast, adventitious embryos are directly formed much less commonly. They are produced on the leaves of a few plant species, and more frequently on nucellar tissue, or on preformed somatic embryos and on the seedling derived from somatic embryos. Method of including regeneration directly in explanted tissues are being discovered in an increasing number of plant species and direct shoot regeneration is an important means whereby several different kinds of plant may be propagated. It should be noted that plants originating from adventitious shoot or embryo meristems have an increased incidence of somaclonal variation. Direct root formation from non-root explants is relatively common for example *Brasisica* (Kartha *et al*., 1974), *Petunia* (Rao *et al*., 1973), *Capsicum* (Gunay and Rao, 1978) and *Abelonoschus esculentus* (Mangat and Roy, 1986).

Shoot meristems formed directly on explanted tissues are often initiated by the commencement of cell division after about 48 h in nutrient culture. Each preliminary

mitosis is rapidly followed by further division, 80 that new adventitious primordia are formed, each comprised of a number of cells. These primordia appear to arise in a polar manner in morphogenetically competent explants or parts of explants, and often tend to be spaced equidistantly. Sometimes each meristem appears to originate from one cell, but this is not always the case. This research are described such meristems can arise from single epidermal cells, in the cotyledons of conifers, meristems arise in subdermal mesophyll (Flinn *et al*., 1988).

During direct organogenesis, adventitious buds may not all be immediately derived from cells of the explant. There is evidence that a single epidermal cell may sometimes give rise to meristematic centres from one of which up to 22 identical shoots (Broertjes *et al*., 1976). The single cell origin of shoots can be confirmed by the high incidence of solid mutant plants, which occurs when suitable organs are subjected to mutagenic treatments. Single cells are mutated and each individual cell may have a different type of mutation from its neighbour. It meristems were initiated from several cells, a high proportion of chimeras would appear. Results such as these have led Broertjes and Van Harten (1978) and Broertjes and Keen (1980) to suggest that directly regenerated adventitious shoot meristems may always be formed from one or a few daughter cells that originate from a single cell. This was confirmed by Nauerby *et al*. (1991). Thomas *et al*. (1979) cautioned that extensive histological work is necessary to tell whether adventitious structures arise from single cells. Following such histological studies, a single cell origin from shoots was found in the basal cells of hairs from *Kohleria* (Geier and Sangwan, 1996). Nevertheless, from a study of shoot regeneration from the leaves of *Saintpaulia* chimeras, Norris and Smith (1981) concluded that the initiation of shoot meristems involves the association and the possible inclusion of cells beneath the epidermis. Adventitious bud meristems which formed between twin bulb scales of onion, arose from many cells in sub-epidermal layers (Falavigna *et al*., 1980). Similarly, shoots on cultured explants of *Nicotiana*

tobacum leaves were found to arise indirectly from nodules at the edges of the explant. The nodules were mainly formed by divisions of palisade mesophyll cells around the edge of the explant where wounding had occurred (Attfield and Evans, 1991). Chimeras have been seen also among the products of adventitious shoot regenerants (Nauerby *et al*., 1991) so confirming the multicellular origin of shoots in some circumstances.

Attfield and Evans (1991) found that roots in the *Nicotiana tobacum* leaf explants were produced directly from the bundle sheath and vein parenchyma, but the number of cells which participated in their origin was not ascertained. That adventitious root formation is also likely to be multicellular in origin can be seen on rooting from shoot segments of *Pisum sativum* and from shoot explants of *Malus domestica* var. Jork 9 (Auderset *et al*., 1994). Roots are seen to arise from parenchymatous cells adjacent to, and between the existing vascular tissue. It is possible that IAA or other PGRs diffuse to the adjacent cells and initiate vascularization and rooting. The procambial tissue is frequency identifiable at the same time as the cells of the initiated root apex. The influence of the existing vascular tissues can be seen on culturing root segments when the cortical parenchymatous cells adjacent to the xylem poles in *Pisum sativum*, will from tracheids prior to the differentiation of the remaining parenchymatous cells (Rana and Gahan, 1982).

1.4 Temporary immersion system (TIS) technology for plant micropropagation

Plant tissue culture is a general term that encompasses a variety of *in vitro* manipulations of plant cells, tissues and organs that direct the de-differentiation of the parental cells into meristematic and embryogenic cells, which then divide and differentiate into plant organs and whole plants. As the piece of the parent plant utilized to initiate the culture the explant is typically small and theoretically every one of its cells has the potential to produce a plant, the end result is the mass multiplication of the parent genotype, known as micropropagation (Ziv, 2000). The morphogenic routes through which cells regenerate

into plants are organogenesis the formation of shoots followed by rooting or of particular organs of interest and embryogenesis the formation of somatic embryos and their germination into complete plantlets, and both can occur via an intervening callus stage. In practice, all micropropagation protocols are established empirically, on a case by case basis, by determining the medium components for example, PGRs, nutrients and environmental conditions for example, light, temperature, each stage of morphogenesis.

Typically, the cultures are supported by a semi-solid substrate which is comprised of a gelling agent. This technique is, therefore, ideal for the clonal multiplication of commercially-important elite genotypes, threatened species and those difficult to propagate through other means. In addition, *in vitro* culture manipulations are now exploited in numerous and ever expanding ways, including germplasm conservation, genetic modification via mutagenesis or genetic engineering, virus elimination, production of secondary metabolites, etc. Details of these techniques and their applications can be found in many books and reviews. As discussed by Mehrotra *et al*. (2007) and Akin-Idowu *et al*. (2009), the advantages of micropropagation over the conventional propagation methods have been accepted decades ago and are now routinely employed by many research laboratories and commercial agriculture (Hamill *et al*., 2009; Snyman *et al*., 2011), horticulture (Akin-Idowu *et al*., 2009), forestry (Watt *et al*., 1997) and other enterprises. However, it has long been recognised that one of the major constraints of such protocols is the need for regular sub-culturing (every 4 to 6 weeks), due to nutrient depletion from the relatively small volumes (10 to 30 mL, depending on container) of semi-solid media utilized, and the associated running and labour expenditure. Scaled-up and automated systems are, therefore, desirable to overcome and/or minimize production costs, increase multiplication rates and reduce the amount of handling during the steps required for micropropagation.

For this purpose, gelling agents are not ideal as, aside from not being inert,

they do not allow for easy automation. Liquid media, on the other hand, provide close contact and uniform access of nutrients to the cultures, they can be renewed without changing the culture vessel, sterilization is possible by ultrafiltration and containers can be larger than those utilized for semi-solid media. However, liquid culture has its disadvantages, including asphyxia and physiological disorders exhibited by the plants. As discussed below, many strategies have been investigated and proposed to overcome the constraints of the protocols that rely on semi-solid media.

1.4.1 Temporary immersion system (TIS)

A TIS is a self-contained sterile environment which operates on liquid nutrient or liquid/air inflow and outflow systems. It is designed for intensive and often scaled-up cultures, and affords maximum opportunity for monitoring and control over micro-environmental conditions for example, agitation, aeration and temperature. The first report on the use of a bioreactor for micropropagation was by Takayama and Misawa (1981) who multiplied Begonia using shake cultures. Today, a large number of different types of reactors have been designed and can be reproduced in-house or can be purchased. Their main differences lie in the types of vessels and the mechanisms that provide culture agitation (non-agitated, mechanical or pneumatically). Most of these were illustrated and described by Etienne and Berthouly (2002) and Paek *et al*. (2001; 2005). The former review presented the automated plant culture system (APCS) with full immersion (Tisserat and Vandercook, 1985) and four partial immersions systems, namely those of Aitken-Christie and Davies (1988), Simonton *et al*. (1991), the RITA® (Alvard *et al*., 1993) and the Twin Flasks (BIT®) (Escalona *et al*., 1999) systems. Paek *et al*. (2001) described the airlift and bubble column-type, balloon-type bubble (BTBB), stirred tank and ebb and flood bioreactors.

Although successes have been reported with all of them, it appears that the choice of bioreactor type lies on the required end product for example, shoot multiplication,

somatic embryos. In some cases, the expertise and resources to build need to be purchased . More recently, Mehrotra *et al*. (2007) discussed the various options for the large-scale plant propagation in bioreactors focusing on those involving submerged cultures but concluded that the ebb and flood types have several advantages, including the independent control of each culture stage. Of all of the available systems, those involving temporary immersion have a variety of characteristics that make them highly suitable for use in semi-automated micropropagation. First advantage is the plant cultures are not constantly immersed in liquid media, which often affects plant growth and morphogenesis negatively. TIS allow for control of contamination, adequate nutrient and oxygen supply and mixing, relatively infrequent subculturing, ease of medium changes and limited shear damage. For these reasons, Ziv (2000, 2005) and Arencibia *et al*. (2008), amongst others, have described TIS as the "most natural tissue-culture approach". A number of different TIS have been utilized successfully in the last seven years for the micropropagation of a variety of plant species of agricultural, medicinal and conservation value. Some of the TIS are patented and others are the result of the ingenuity of researchers who may not have the funds to purchase them.

The Twin-Flask system (BIT®) of Escalona *et al*. (1999) consists of a container for growing plants and a reservoir for the liquid medium. When a solenoid valve is opened and compressed air is turned on, the medium is forced into the first flask, immersing the plants. The process is reversed when another solenoid valve is opened and air pressure forces the medium back into the original reservoir.

The RITA® system (recipient for automated temporary immersion) (Alvard *et al*., 1993) is an apparatus made up of an upper compartment containing the explants and lower compartment which contains the liquid culture medium. They are linked together so that when an overpressure is applied to the lower compartment, the medium is push into the upper one; when the overpressure drops, the liquid medium returns to the lower

compartment. Consequently, the cultures in the upper compartment are temporarily immersed when the upper compartment is flooded with the liquid medium, the frequency and time period of which can be regulated. Stanly *et al*. (2010) applied this principle to a reusable Nagene® polysulfone filtration system by connecting each of the two compartments with a tube fitted with a 22 µm filter, through which pressure was applied to push the culture medium onto the upper compartment that housed the explants. The outlet on the top of the vessel allowed for pressure to escape and the air entering the vessel was filtered through a sterile syringe filter. A similar set-up to that of the RITA® that uses Plantima containers has recently become available (Yan *et al*., 2010).

The bioreactor of immersion by bubbles (BIB®) system developed by Soccol *et al*. (2008) has an interlinked system with tubes of flexible rubber that provide the cultures with air and nutrient solution by bubbling. The apparatus has two glass compartments divided transversally by a porous plate. On the top (larger compartment), stainless steel tripods and mesh are inserted to support the cultures.

The successes and benefits of the described semi-automated TIS, for the mass multiplication of plants, were comprehensively reviewed by Etienne and Berthouly (2002) and Paek *et al*. (2001; 2005). At that time, it was abundantly clear that the technology offered new ways to achieve high plantlets yields *in vitro*, at low cost, suitable for research and commercial activities. The aim of the present review is to assess the subsequent progress on the uses of TIS, and the realization of such promise, since those publications.

1.4.2 Factors affecting organ and plantlet yield in TIS

The strategy was to utilize *in vitro* explants to initiate TIS so as to eliminate the problem of microbial contamination and consequent culture losses. However, particularly in woody species, the explants (nodal explants, buds) carry endogenous bacteria or fungi that proliferate very quickly once exposed to the liquid medium. This

together with the relatively large number of explants placed in each vessel is the main cause of TIS *Eucalyptus* spp. culture losses (McAlister *et al*., 2005; Watt *et al*., 2006). Hence, in large commercial activities, such as forestry, the cost implications of such incidents can be extremely serious. There are a number of approaches (for example, serological techniques, protein and genetic profiling) to dealing with endogenous and latent contamination, but the majority require specialised instrumentation and labour as reviewed by Herman (2004). The most commonly- employed tactic is treating the explants with and incorporating antibiotics and biocides such as PPM™ (Plant Cell Technology, Washington, DC) in the culture media. Although often successful (McAlister *et al*., 2005; Luna *et al*., 2008), antibiotics are expensive, they have optimum pH conditions and degrade quickly.

Another option, particularly when dealing with explants from parent plants grown in the field, is to screen them on a semi-selective microbial medium such as that formulated by Viss *et al*. (1991). This has proven effective for the production of axenic start-up cultures of eucalyptus buds in RITA® vessels (Watt *et al*., 2006). In a similar approach, Mordocco *et al*. (2009) employed semi-solid SmartSett® shoot induction medium to obtain contaminant-free sugarcane explants for multiplication in RITA®. Micropropagation success in TIS depends on; the volumes of the culture container and liquid medium in relation to explant biomass at initiation and subsequent culture stages, the immersion regimes to which the cultures are subjected and the effect that each of these parameters has on the others. Given the various types and sizes of TIS, as well as types of cultures, there is a wide range of published inoculum densities, for example, 10 pineapple buds per 300 mL medium in 1 L flasks (da Silva *et al*., 2007). Although biomass of the explants is usually reported as part of the description of the protocol used, only a few authors have reported on the optimization of the explant biomass for TIS initiation. In this regard, the highest multiplication rate for *Eucalyptus* spp. was obtained when cultures were started with 50 buds per RITA® vessel (McAlister *et al*., 2005). For the production of potato microtubers, the best ratio was 60

explants in 3.5 L media (Perez-Alonso *et al*., 2007) and for the multiplication of plantain it was 5 intact shoots and 150 mL media (Cejas *et al*., 2011).

A commonly-encountered problem in tissue culture is hyperhydricity of the cultured material, it is more prevalent in liquid culture as a result of the constant, partial or temporary immersion of the explants and often leads to necrosis (Berthouly and Etienne, 2005). Consequently, in TIS the duration and frequency of the immersion are the most decisive parameter for successful micropropagation, as they influence nutrient and water uptake and consequently hyperhydricity of the cultured material. Immersion regimes vary greatly depending on the species under study, the TIS employed and the route of morphogenesis. Regarding the latter, evidence has accumulated in support of the proposal by Teisson and Alvard (1995) that frequent but short immersion duration cycles stimulate somatic embryogenesis and eliminate embryo hyperhydricity. For example, Albarrán *et al*. (2005) showed that changing from 15 min every 4 h to 1 min every 4 h decreased hyperhydricity and increased embryo conversion of coffee somatic embryos and Gatica-Arias *et al*. (2008) employed an even longer resting time (1 min every 8 h). Similar results were reported for cacao when that same immersion regime was utilized (Niemenak *et al*., 2013). Shoot hyperhydration is usually found to be lower in TIS than in semi-solid and liquid media and most of the recent publications continue to uphold this notion (Yan *et al*., 2010). Increasing the resting time between immersions from 1 min every 12 h to 1 min every 72 h, combined with reduced nutrient supply, decreased hyperhydricity significantly in sugarcane plantlets produced in RITA® (Snyman *et al*., 2011). Similarly, lower nutrient supply and sucrose levels, combined with a 2 min every 12 h immersion regime successfully diminished hyperhydration in *E. globulus* shoots produced in a twin-flask system (González *et al*., 2011). Various researchers attribute the beneficial effect of TIS on the elimination of hyperhydricity and increased propagation yields, compared with semi-solid and liquid protocols, to the renewal of its headspace with the surrounding air

(Zobayed, 2005). According to Roels *et al*. (2005, 2006) TIS can prevent the accumulation of CO_2 and C_2H_4 that occurs above the semi-solid medium and has detrimental effects on the shoots in culture.

1.5 The application of molecular marker

In plant genetics and breeding studies, DNA-based assays, and especially molecular markers, are known to be efficient tools for genetic diversity assessment, molecular ecology studies, gene mapping as well as marker-assisted selection (MAS) (Feng *et al*., 2009). Among all the available molecular markers, simple sequence repeats (SSR) are still among the most favored, due to their many desirable attributes, which include hyper variability, wide genomic distribution, co-dominant inheritance, a multi-allelic nature and chromosome specific location. In addition, they are easily assayed using PCR (Powell *et al*., 1996). Currently, SSR also appear to be the most promising molecular marker systems for understanding oil palm population genetic structure (Singh *et al*., 2008). Furthermore, SSR markers which are highly transferable across taxa are advantageous as they save time and cost in developing SSR markers for members of taxa that have not been extensively studied. These SSR markers are also useful tools for comparative genetic studies within the genus. In oil palm, *E. guineensis*-based SSR markers have been used to construct genetic maps (Billotte *et al*., 2005; Billotte *et al*., 2010), and are also actively used to characterize germplasm collections (Teh, 2010). The advantages of SSR marker is highly polymorphic and informative, co-dominant, technically simple, reproducible and relatively inexpensive (Qu *et al*., 2012). SSR marker was used to detect somaclonal variation in oil palm (Thawaro and Te-chato, 2009; Sanputawong and Te-chato, 2011; Khawnium and Te-chato, 2011), cotton (Jin *et al*., 2008) and pine (Marum *et al*., 2009). 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 used this technique for analyze somaclonal variation in potato plantlets regenerated from callus.

1.6 Objectives

1.6.1 To compare performances of solid medium, liquid medium and TIS on embryogenic callus proliferation of oil palm

1.6.2 To develop culture medium for induction of somatic embryo directly from roots of oil palm plantlets in vitro and origin of these somatic embryo

1.6.3 To develop culture medium for somatic embryo induction and plantlet regeneration from embryogenic callus of oil palm

1.6.4 To assess genetic variability of *in vitro* embryogenic callus from TIS culture by SSR marker

CHAPTER II

Proliferation of Embryogenic Callus of Oil Palm Using Different Culture Systems:

Comparison of Solid Medium, Liquid Medium and TIS on Embryogenic Callus Production of Oil Palm

Introduction

Oil palm is a diploid monocotyledon belonging to the family Areaceae. It is an economically important product, as it is the source of palm oil, the most traded vegetable oil in the international market and it is increasingly used in the food industry (Corley and Tinker, 2003). Given the increasing economic value of oil palm, micropropagation of this tropical crop has been studied to develop oil palm propagation with a view to the commercial distribution of elite clones in order to increase oil production (de Touchet *et al*., 1991). Clonal propagation of oil palm has been studied for many years as a potential way to develop high- yielding collections while circumventing the long generation time required with traditional breeding techniques. In 1991, the first generable oil palm friable embryogenic tissues were obtained from culturing of zygotic embryos of immatureand successfully generated embryogenic cell suspension cultures (Teixeira *et al*., 1995). Although these two protocols were successful for establishment of single somatic embryos from embryogenic cell suspension cultures, the regeneration rate into plantlet was poor. This issue was overcome by Aberlenc-Bertossi *et al*. (1999), who developed a protocol to improve the quality of shoot formation. The first result obtained from suspension culture-derived clones encourages and the researchers to field trail (Rival *et al*., 2001). Most of these protocols used solid medium, and their efficiency is rather low considering that the multiplication rate for most protocols takes more than 60 days while liquid medium with agitation takes shorter period during the multiplication stage (Lee-Espinosa *et al*., 2008). TIS, among other advantages, allows for the semi-automation of the culture process, facilitates medium renewal, and also combines aeration and explant immersion with programmed duration and frequency of immersion, thus reducing hyperhydricity of the plant and increasing survival rate of plants or somatic embryos (Levin and Tanny, 2004). The TIS has been used in the micropropagation of several species including *Malus* sp.

(Zhu *et al*., 2005), *Eucalyptus* spp. (McAlister *et al*., 2005), *Saccharum* spp. (Mordocco *et al*., 2009) and *Quercus robur* (Mallon *et al*., 2012).

Sucrose is the most common carbon source used in plant cell, tissue and organ culture. Culture media with 3% sucrose have been commonly used. (Drapeau *et al*., 1987) Sucrose acts during plant tissue culture as a fuel source for sustaining photo-mixotrophic metabolism, ensuring optimal development, although other important roles such as carbon precursor or signaling metabolite have more recently been highlighted (Martinez *et al*., 1993). Sucrose also supports the maintenance of osmotic potential and conserves water in cells. However, high sucrose concentration in culture media restricts the photosynthetic efficiency of cultured plants by reducing the levels of chlorophyll, key enzymes for photosynthesis (Hazarika, 2006). The objective of the present study was to compare performances of solid medium, liquid medium and TIS on embryogenic callus proliferation of oil palm.

Materials and methods

Plant materials

Immature fruits of oil palm, dura×pisifera (D×P) at 3 months after pollination (MAP) was kindly provided by Golden Tenera Company were used as explants for germination of seedlings. Immature zygotic embryos were aseptically excised and cultured on MS (Murashige and Skoog) medium supplemented with 2.5 mg/L dicamba, 3% sucrose and 200 mg/L ascorbic acid. The medium was solidified with 0.75% agar and adjusted to pH 5.7.

Embryogenic callus of oil palm was induced on 2.5 mg/L dicamba containing MS medium and proliferated on the same medium supplemented with 0.1 mg/L dicamba, 3% sucrose and 200 mg/L ascorbic acid (EC medium). The medium was solidified with 0.75% agar and adjusted to pH 5.7 before autoclaving at 1.05 kg/cm^2, 121 °C for 15 min. The cultures were placed under 15 $\mu mol/m^2/s$ light illumination, 14 h photoperiod at 28±1 °C and sub-cultured after one month of culture.

Description of the automated TIS

The TIS consisted of two containers, one for growing plant cells and a reservoir for liquid medium. The two containers were connected by silicones and glass tubes. In each case, the airflow was sterilized by passing through 0.2 μm millipore filters. Air pressure from an air compressor pushed the medium from one container to the other to immerse the plants completely. The air flow was reversed to withdraw the medium from the culture vessel. Electronic timers control the frequency and length of the immersion period regulated by three-way solenoid valves.

Evaluation of the different culture systems on growth ratio of EC

Three culture systems; solidified culture medium, liquidified culture medium with agitation at 110 rpm and TIS using the automated system were performed; a 0.5 gram fresh weight (gFW) of embryogenic callus were used as initial inoculum for solidified and liquidified culture medium. The volume of culture medium was (EC medium) was 25 mL containing in 125 mL Erlenmyer flask. For the TIS, 2 gFW of EC were placed in 500 mL Erlenmyer flask containing 150 mL liquid EC medium. The medium was supply to EC for 3 min and withdrawn to let EC dry for 4 h. After one month of culture growth ratio of EC was recorded and statistically compared among the three different culture systems using completely randomized design (CRD) with 3 replicates (each replicate consisted of 0.5 or 2 gFW of EC). Mean values among treatments were separated by Duncan's multiple range test (DMRT).

Evaluation of different concentrations of sucrose on growth ratio of EC in TIS system

EC derived from above experiment at 2 gFW was cultured in TIS system supplemented with 150 mL of liquid EC medium and the medium was supplemented with five different concentrations of sucrose; 3.00, 3.25, 3.50, 3.75 and 4.00%. EC was immersed in EC medium for 3 min followed by removal of the medium to let the EC dry for 4 h. After one month of culture growth ratio of EC was recorded and statistically compared among the different concentrations of sucrose using CRD with 3 replicates (each replicate consisted of 2 gFW of EC). Mean values among treatments were separated by DMRT.

Evaluation of the different drying periods on growth ratio of EC in TIS system

For the TIS, two gram fresh weight of EC were cultured in 150 mL liquidified EC medium containing in 500 mL Erlenmyer flask. The culture flask was connected with nutrient supply flask and let liquid medium flow into plant cell culture flask by the volume and time regulated by timer. By this system, 150 mL of culture medium was feed to culture

flask containing 2 gFW of EC and immersed for 3 min. The culture medium was then drawn back to nutrient supply flask to let the callus dry for four periods; 4, 8, 12 and 16 h. After one month of culture growth ratio of EC was recorded and statistically compared among four different periods of drying CRD with 3 replicates (each replicate consisted of 2 gFW of EC). Mean values among treatments were separated by DMRT.

Effect of culture medium volume per flask on growth ratio of EC in TIS system

To evaluate the effect of volume of culture medium per cultured EC for the TIS, five volumes of liquidified medium; 50, 100, 150, 200 and 250 mL per flask were tested. In all cases, EC was immersed in those volumes of EC medium for 3 min and dry by optimum period obtained from the previous experiment. After one month of incubation growth ratio of EC was recorded and statistically compared among the volume of culture medium using CRD. Each volume of the medium consisted of three flasks. Mean values among treatments were separated by DMRT.

Results and Discussion

Evaluation of the different culture systems

To evaluate the three culture systems, solid culture medium, liquid culture medium and the automated TIS, one vegetative growth parameters of oil palm were measured after one month of culture. The results revealed significant differences among the three culture systems evaluated, with the best growth ratio achieved with TIS at 5.14 followed by the liquid medium (4.46) and solid medium (2.51) (Table 2.1, Figure 2.1). Therefore, the greatest impact of the three culture systems were substantial increase in the growth ratio of oil palm using a liquid medium system, compared with that of the solid medium. This increase was the greatest when the TIS was used. Micropropagation in liquid culture medium increases nutrient uptake and promotes growth, but hyperhydricity is

commonly occured. Continuous contact of plant tissues with the liquid medium is the source of hyperhydricity (Debergh *et al*., 1991). TIS, which combines the advantages of solid and liquid cultures, have been developed successfully for many plant species and offers significant advantages including an efficient supply of nutrition and automation at relatively low cost (Yang and Yeh, 2008).

Table 2.1 The effect of different culture systems on growth ratio of EC after one month of culture.

Culture system	Growth ratio±SD
Solid	2.51±0.26
Liquid	4.46±0.28[a]
TIS	5.14±0.28[a]
F-test	*
C.V.(%)	21.69

* : significant difference at $p \leq 0.05$

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

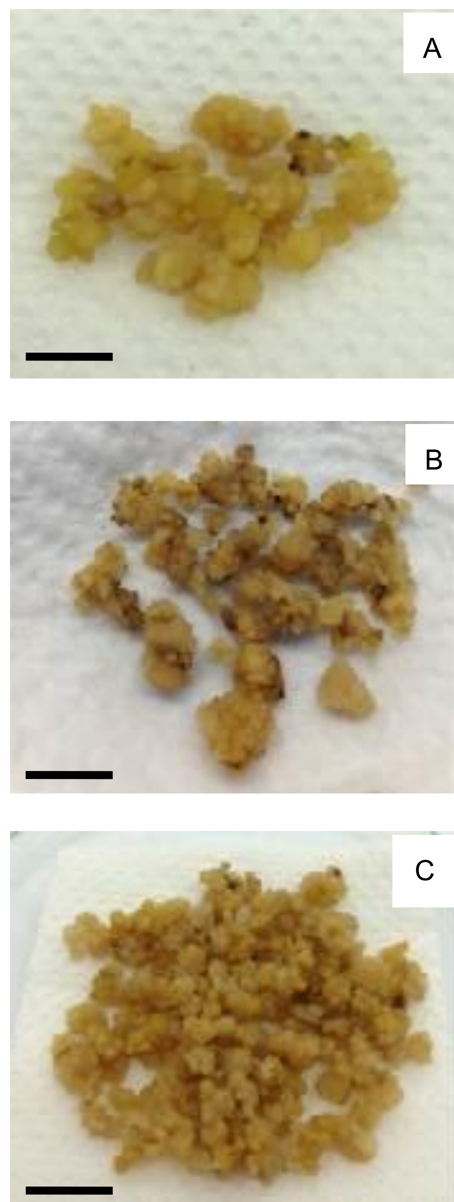

Figure 2.1 Embryogenic callus obtained from different culture system on EC medium supplemented with 0.1 mg/L dicamba, 3% sucrose and 200 mg/L ascorbic acid, with solid culture medium (A), liquid culture medium (B) and TIS system (C) after one month of culture (bar = 1 cm).

Sreedhar (2009) found no differences in the multiplication rate for *Vanilla planifolia* among solid medium, complete immersion and TIS with Growtek™ system. This author obtained 2.46 shoots/explant and double the shoot elongation and the biomass with the TIS over 5 weeks. This maybe because the author used explants that had been cultured for 10 years and the shoot tips were not removed to allow for the formation of new shoots. Similarly, for banana (*Musa* spp.), Farahani and Majd (2012) achieved double the shoot formation rate relative to that obtained using solid or liquid media. Arencibia *et al.* (2013) also double the shoot multiplication rate in raspberry (*Rubus* spp.) using a Temporary immersion bioreactor (TIB). Hempfling and Preil (2005) reported the achievement of the shoot multiplication rate in *Phalanenopsis* spp. that TIB increased around fivefold over that obtained using solid medium. Steinmacher *et al*. (2011) also reported that the use of a TIS greatly improved the number of somatic embryos obtained supsequent to germination of seedlings of peach palm (*Bactis gasipaes*). Yan *et al.* (2010) obtained a greater shoot multiplication in monk fruit (*Siraitia grosvenorii*) using the Plantima® system in comparison with solidified or liquidified medium.

The effect of different concentrations of sucrose on growth ratio of EC in TIS system

EC cultured on EC medium supplemented with 3.75% sucrose gave the highest growth ratio of EC at 7.92, significant different with another concentration, followed by sucrose at concentration of 4.00, 3.50, 3.25 and 3.00% which gave the growth ratio of EC proliferation at 3.66, 1.02, 0.46 and 0.00, respectively (Table 2.2, Figure 2.2). The proliferation of already formed EC may be inhibited by high levels of sucrose in the medium. Indeed, concentration of sucrose above 3.75% suppress proliferation of callus of various plant (Hildebrandt and Riker, 1953). Low concentrations of sucrose favour the initiation of numberous shoot in tobacco callus and depress the growth of callus (Barg and Umiel, 1997). Sucrose is another factor affecting physiology processes in plant growth and development. Among those sucrose is commonly used as carbon and energy sources in

plant tissue culture (Murashige and Skoog, 1962; White, 1943 and Gamborg, 1970) which influences upon plant regeneration (Chen and Chang, 2004). In addition, sucrose is primary source known for reliable in induction and development of flowers *in vitro*. The presence of carbon sources in culture medium is necessary for floral stimulation (Singh *et al*., 2006).

Table 2.2 The effect of various concentrations of sucrose containing medium in TIS on growth ratio of EC after one month of culture.

Concentrations of sucrose (%)	Growth ratio±SD
3.00	0.00±0.00[c]
3.25	0.46±0.22[c]
3.50	1.02±0.51[c]
3.75	7.92±0.37[a]
4.00	3.66±0.74[b]
F-test	*
C.V.(%)	29.46

* : significant difference at $p \leq 0.05$

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

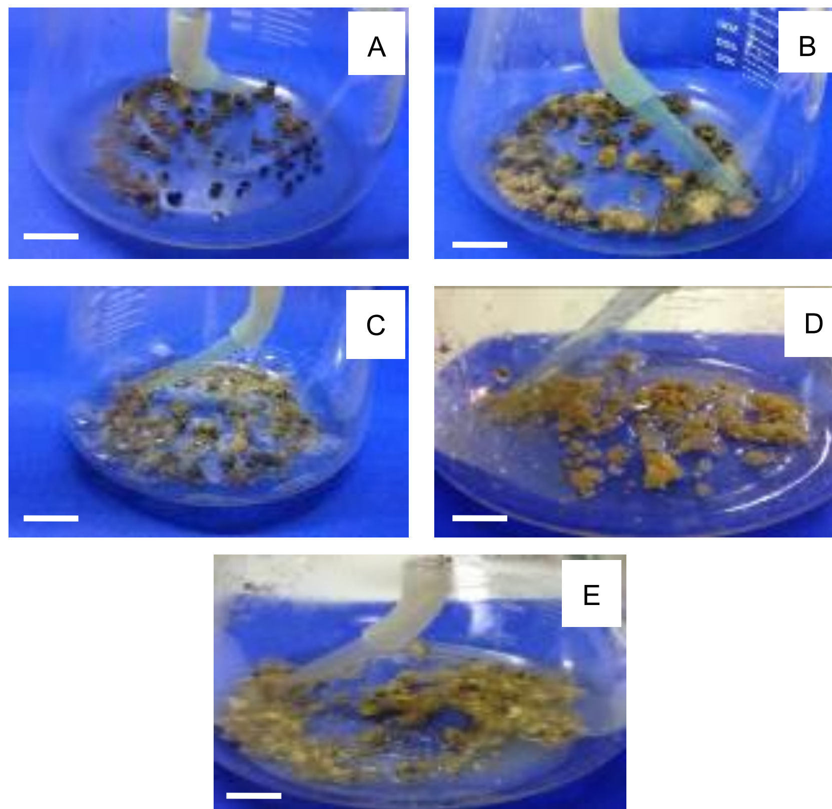

Figure 2.2 EC of oil palm culture in TIS system with EC medium with various concentrations of sucrose after one month of culture, 3.00% sucrose (A), 3.25% sucrose (B), 3.50% sucrose (C), 3.75% sucrose (D) and 4.00% sucrose (E) (bar = 1 cm).

Determination of drying period on growth ratio of EC in TIS system

The effect of different drying period after immersion EC for 3 min in TIS on proliferation or growth ratio of EC gave different results. Immersion EC in liquidified EC medium for 3 min followed by drying period for 8 h gave the highest growth ratio of EC at 10.07 significant difference ($p \leq 0.05$) with drying period at 4, 12 and 16 h which gave growth ratio of EC at 6.19, 3.79 and 3.62, respectively (Table 2.3, Figure 2.3). In addition, characteristics of the EC were quite differences. All ECs obtained from dry period for 4 h were yellow in color and friable which could be separated easily (Figure 2.3A). In the case of drying period at 8 h, EC increased was observed in approximately, 10.07 of the EC formed yellow then turn brown color and compact callus (Figure 2.3B). From this

experiment it was concluded that the optimal results were achieved using a system with 3 min immersions every 8 h for proliferation ration of oil palm EC. The temporary immersion cycle is a key factor which influences the success of the micropropagation process by influencing the uptake of nutrients, plant growth regulators and water (Roels *et al*., 2005). As the drying period decreased, the obtaining plantlets were less vigorous, suggesting that oil palm is a nutrient demanding species.

In the micropropagation of banana (*Musa* spp.) using a TIB, Roels *et al*. (2005) found no significant differences in shoot production per explant with 4 min immersions every 3, 5 and 7 h but in the later treatment, shoots were longer. In habanero chili (*Capsicum chinense*), increase in the multiplication rate was obtained by using the Modular Temporary Immersion Bioreactor (BioMINT^{TM}) with 2 min immersion every 8 h (Bello-Bello *et al*., 2010). Sreedhar (2009) obtained a maximum proliferation of 2.42 shoots/explant with 30 min immersion every 8 h using the Growtek^{TM} system of V*anilla* spp. Furthermore, hyperhydricity was observed in the plantlets generated with 45 min immersion every 8 h. In this present study, different periods of immersion were not investigated. However, the best response was obtained from 3 min immersion every 4 h. This condition may prevent hyperhydricity and callus growth on plantlets and result in production of vigorous plant.

Table 2.3 The effect of drying period in TIS on growth ratio of EC after one month of culture.

Drying periods (h)	Growth ratio±SD
4	6.19±0.52[b]
8	10.07±0.58[a]
12	3.79±0.27[c]
16	3.62±0.50[c]
F-test	*
C.V.(%)	14.13

* : significant difference at $p \leq 0.05$

Mean values followed by the same letter with in column are not significantly different according to DMRT

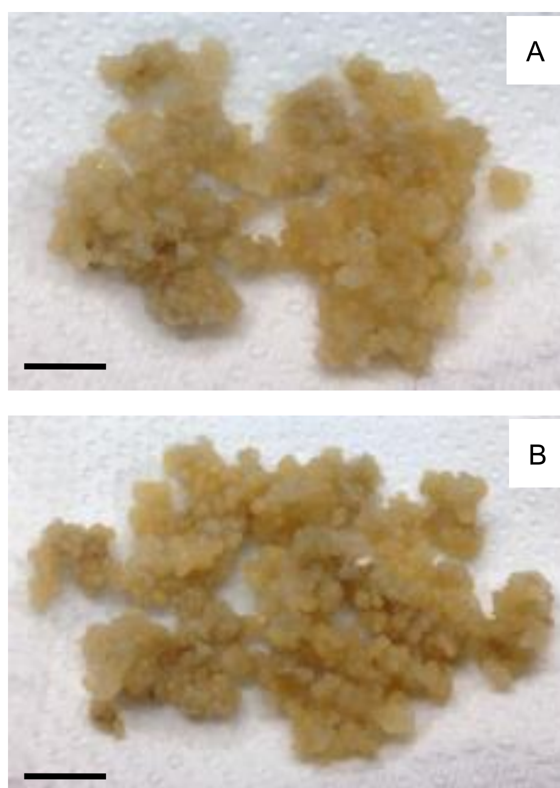

Figure 2.3 EC of oil palm culture in TIS system with EC medium after one month of culture, 3 min immersion every 4 h (A) and 3 min immersion every 8 h (B) (bar = 1 cm).

Volume of culture medium per flask on growth ratio of EC in TIS system

Our experiments also determined that the smaller volume (150 mL) of culture medium per explant increased the highest growth ratio at 11.19, significant difference ($p \leq 0.05$) with volume of 100, 250, 50 and 200 mL, which produced growth ratio at 7.22, 4.66, 4.30 and 4.22 respectively (Table 2.4, Figure 2.4). In banana (*Musa* spp.), it was found that 30 mL of medium per explant was the best volume for the maximum multiplication rate (Roels *et al*., 2005). Jin *et al*. (2013) reported that propagation of grape (*Vitis vinifera*) rootstock using the air lift bioreactor doubled the plantlet biomass using 30, 40 and 76 mL of medium per explant in comparison with 25 mL. According to Lorenzo *et al*. (1998), larger volumes of culture medium produced lower multiplication rates in sugarcane (*Saccharum* spp.). A possible explanation is that explants excrete some compounds that promote shoot formation, which would be diluted when large volumes of culture medium are used.

Table 2.4 The effect of volume of culture medium in TIS on growth ratio of EC after one month of culture.

Volume of medium (mL)	Growth ratio±SD
50	4.30±0.61[b]
100	7.22±0.29[a]
150	11.19±0.34[c]
200	4.22±0.37[c]
250	4.66±0.51[b]
F-test	*
C.V.(%)	12.06

* : significant difference at $p \leq 0.05$

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

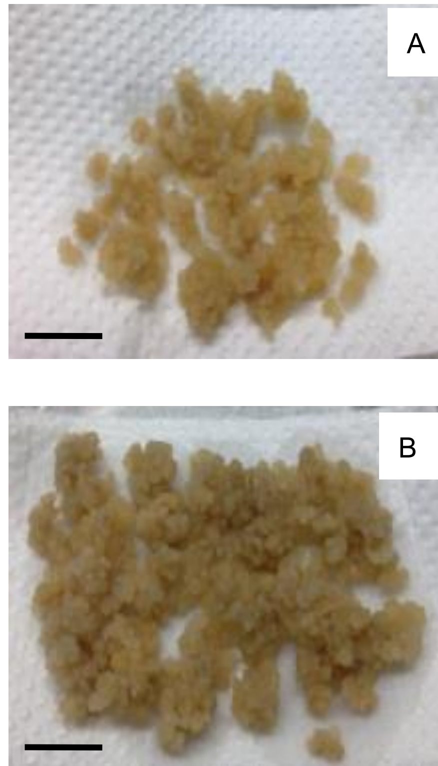

Figure 2.4 EC of oil palm culture in TIS system with different volumes of EC medium after one month of culture. (A) volume of culture at 50 mL, (B) 150 mL (bar = 1 cm).

CHAPTER III

Micropropagation through Somatic Embryo Induction Directly from Root of Oil Palm Plantlets:

Effect of Dicamba on Induction of Somatic Embryo and Plant Regeneration

Introduction

Oil palm is the most productive oil crop with yield of up to 5-7 tons of palm oil/ha/year (Rajesh *et al*., 2003). Propagation of oil palm through SE is a promising regenerative route, as this morphogenetic pathway may increase the number regenerated plantlets in comparison with organogenesis (Steinmacher *et al*., 2007). SE is the developmental process by which somatic cells develop into structures that resemble zygotic embryos through an orderly series of characteristic embryological stages without fusion of gametes (Jimenez, 2005). This developmental route can be induced by many factors such as explants, culture media, plant growth regulators and culture conditions (Thuzar *et al*., 2005). Types and concentrations of PGRs in the culture media can greatly affect somatic embryo or somatic embryogenesis induction of oil palm tissue culture. Regeneration of culture cells is essential for crop improvement through biotechnology. Among the different auxin 2,4-D, a synthetic auxin is the most widely used for oil palm callus induction at a concentration of 80-100 mg/L. A much of lower concentration of 2,4-D was applied as the sole sources of plant growth regulator in the regeneration medium to induce regeneration of cultured oil palm cells (de Touchet *et al*., 1991). Dicamba, another plant growth regulator and used as selective systemic herbicide against annual, perennial broad-leaved weeds and bush species (Pena-Ramirez *et al*., 2011) is also effective on induce callus formation. Dicamba has been reported to be an effective auxin for increasing a large number of somatic embryos (Te-chato *et al*., 2003a). NAA has also been reported to induce callus formation but high concentration at 40 mg/L was required, unfortunately, development of somatic embryos through embryogenesis was not reported (Te-chato, 1998b). The main effect of NAA was reported to induce the formation of a primary ortho-gravitropic root comparable of the developed during *in vitro* germination of oil palm seeds (Jourdand and Rey, 1997).

Another important aspect for adequate morphogenic response is the culture media used (Ainsey *et al*., 2000). The different culture media have been reported for culturing calli with positive results in woody plants (Vangadesan *et al*., 2000). Among those media MS (Murashige and Skoog) (Murashige and Skoog, 1962) medium was popular used for callogenesis and subsequent organogenesis of *Alnus sinuate* (Corredoira *et al*., 2003), other medium which was widely used in woody plant species is the Woody Plant (WPM) (Lloyd and McCown, 1980). This culture medium produced the best shoot growth for the micropropagation of the 'Bluecrop' highbush blueberry (Takuya *et al*., 2008). Oil palm culture medium (Heedchim, 2014), a mixture of basic salt between MS and WPM medium was reported to be superior to shoot multiplication and elongation in oil palm tissue culture.

Tissue culture techniques have been effectively used to assist the genetic improvement of several crop species. Regeneration of plants from root tissue is not only useful for rapid propagation of clones through organogenesis, but also generated somatic embryos from intact root-derived callus of *Prunus incise* × *P. serrula* and horse-chest nut (*Aesculus hippocastanum* L.) (Rosa *et al*., 1986). Moreover, embryoid structures were also induced on nodular callus developed from the roots of micropropagated cherry rootstock (*P. avium* × *P. pseudocerasus*) plantlets (James *et al*., 1984). Russell and McCown (1986) developed highly competent embryogenic and organogenic systems based on the formation of nodules on the roots induced from vitro shoots of *Populus*. Histological studies describing the origin and different stages of *in vitro* morphogenesis have contributed significantly to the understanding and optimization of various regeneration systems (Rosa and Dornelas, 2012). Thus, the objective of the present study was to develop culture medium for induction of somatic embryo directly from roots of oil palm plantlets *in vitro* and origin of these somatic embryo.

Materials and methods

Plant materials

Immature fruits of oil palm of breeding material from dura×pisifera (D×P) crosses at 3-months after pollination (MAP) was provided by Golden Tenera Company and used as explants for germination of seedlings. Immature zygotic embryos (IZEs) were aseptically excised and cultured on MS medium supplemented with 2.5 mg/L dicamba, 3% sucrose and 200 mg/L ascorbic acid. The medium was solidified with 0.75% agar, adjusted to pH 5.7 before autoclaving at 1.05 kg/cm^2, 121 °C for 15 min.

Influence of culture media and various concentrations of dicamba, 2,4-D and NAA on SE formation frequency and number of SEs

Plantlets were cultured on different media (MS, WPM or OPCM) (Table 3.1) supplemented with various concentrations of dicamba, 2,4-D or NAA (0.25, 0.50, 0.75 and 1.00 mg/L), 3% sucrose and 200 mg/L ascorbic acid. The medium was solidified with 0.75 % agar and adjusted to pH 5.7 before autoclaving at 1.05 kg/cm^2, 121 °C for 15 min. The cultures were placed under light conditions at 15 $\mu mol/m^2/s$, 14 h photoperiod at 28±1 °C and sub-cultured after 1 month of incubation. Experiment was designed by using completely randomized design (CRD) with 4 replicates (each replicate consisted of 5 plantlets). The percentage of SE formation and the number of SEs developed directly from in vitro-seedling roots were recorded. Mean values among treatments were separated using Duncant's multiple range test (DMRT).

Table 3.1 Components of different culture media used for culturing of oil palm plantlets.

Elements	Concentration (mg/L)		
	MS	WPM	OPCM
Macronutrients			
NH_4NO_3	1,650.000	400.000	1,025.000
KNO_3	1,900.000	-	950.000
KH_2PO_4	170.000	170.000	170.000
$CaCl_2.2H_2O$	440.000	96.000	268.000
$MgSO_4.7H_2O$	370.000	-	185
Micronutrients			
KI	0.830	-	0.415
K_2SO_4	-	990	495.000
H_3BO_3	6.200	6.200	6.200
$MnSO_4.H_2O$	16.900	16.900	16.900
$ZnSO_4.7H_2O$	10.600	8.600	9.600
$CuSO_4.5H_2O$	0.025	6.250	3.138
$Na_2MoO_4.2H_2O$	0.250	0.250	0.250
$CuCl_2.6H_2O$	0.025	-	0.0125
$FeSO_4.7H_2O$	27.800	27.800	27.800
Na_2EDTA	37.300	37.300	37.300
Organics			
Myo-inositol	100.000	100.000	100.000
Nicotinic acid	0.500	0.500	0.500
Pyridoxine HCl	0.500	0.500	0.500
Thiamine HCl	0.100	0.100	0.550
Glycine	2.000	2.000	2.000
Sucrose (g)	30.000	30.000	30.000
Agar (g)	7.5000	7.5000	7.5000
pH	5.7	5.7	5.7

Histological analysis

For histological observation of somatic embryo derived from root cultured on OPCM medium supplemented with 0.5 mg/L NAA, 200 mg/L ascorbic acid, 3% sucrose and 0.75% agar was fixed by immersing into formalin : acetic acid : alcohol solution (FAA, 1 : 1 : 9 v/v) at room temperature for 24 h. The samples were dehydrated through an ethanol series at 30, 50, 70, 85, 95 and 100%, respectively at 2 h for each concentration. The samples were embedded in Paraffin wax at 60 ºC, sectioned with rotary microtome at 8 µm and fixed on glass slides. The sections were de-waxed in xylene for 10 min, stained with Delafield' s Hematoxylin and Johansen's Safranin solution for 15 min (Johansen, 1940) and observed under light microscope (Olympus CKX 41).

Results and Discussion

Influenze of culture media and various concentrations of dicamba, 2,4-D and NAA on SE formation frequency and number of SEs

Different PGRs gave the different responses in frequency and number of SE formation. Plantlet cultured on MS medium without plant growth regulator, could not produce SE (Figure 3.1A). Whereas, MS medium supplemented with 0.5 mg/L NAA resulted in the highest percentage of SE formation at 35 and average number of SEs at 0.45 SEs/root (Table 3.2). SEs derived from root directly had creamy to green color (Figure 3.1B).

NAA at concentrations of 0.50 mg/L gave the highest number of SEs at 0.7 SEs/root, followed by 2,4-D at 0.25 mg/L which gave the average number of SEs at 0.6 SEs/root . However, percentage of SE formation obtained from 0.5 mg/L NAA and 0.25 mg/L 2,4-D containing medium gave equal result at 50 (Table 3.2). Those SEs had yellow to green in color (Figure 3.1C).

SEs was successfully germinated into seedlings, shoot with root. The best results obtained from the present study were root formation from SE just after emergence of shoot. SEs being initiated on OPCM supplemented with 0.5 mg/L NAA after 2 months of culture. The highest average number of SEs at 1.20 SEs/root and percentage of SE formation at 80 was obtained in OPCM (Table 3.2). SEs developed on this medium was yellow to green in color and germinated directly into shoot and root, either singly or in group (Figure 3.1D).

Table 3.2 Effects of culture media supplemented with different concentrations of dicamba, 2,4-D or NAA on the number of SEs and percentage of SE formation after culturing for one month.

Plant growth regulators	Concentrations (mg/L)	Avg. no. of SEs±SD			SE formation (%)		
		MS	WPM	OPCM	MS	WPM	OPCM
Dicamba	0	0.00±0.00	0.00±0.00	0.00±0.00	0	0	0
	0.25	0.20±0.00	0.35±0.05	0.30±0.06	20	25	25
	0.50	0.00±0.00	0.20±0.00	0.25±0.05	0	20	20
	0.75	0.00±0.00	0.00±0.00	0.00±0.00	0	0	0
	1.00	0.20±0.00	0.00±0.00	0.25±0.05	0	0	20
2,4-D	0	0.00±0.00	0.00±0.00	0.05±0.05	0	0	5
	0.25	0.00±0.00	0.60±0.00	0.50±0.06	0	50	30
	0.50	0.20±0.00	0.40±0.00	0.25±0.05	20	20	20
	0.75	0.25±0.05	0.00±0.00	0.05±0.05	25	0	5
	1.00	0.20±0.00	0.00±0.00	0.15±0.05	20	0	15
NAA	0	0.00±0.00	0.00±0.00	0.00±0.00	0	0	0
	0.25	0.00±0.00	0.40±0.00	0.30±0.06	0	40	25
	0.50	0.45±0.05	0.70±0.06	1.20±0.08	35	50	80
	0.75	0.15±0.00	0.20±0.00	0.90±0.05	15	20	55
	1.00	0.35±0.05	0.20±0.05	0.70±0.05	30	20	50
F-test		ns			ns		
C.V. (%)		38.27			25.12		

ns: not significant difference

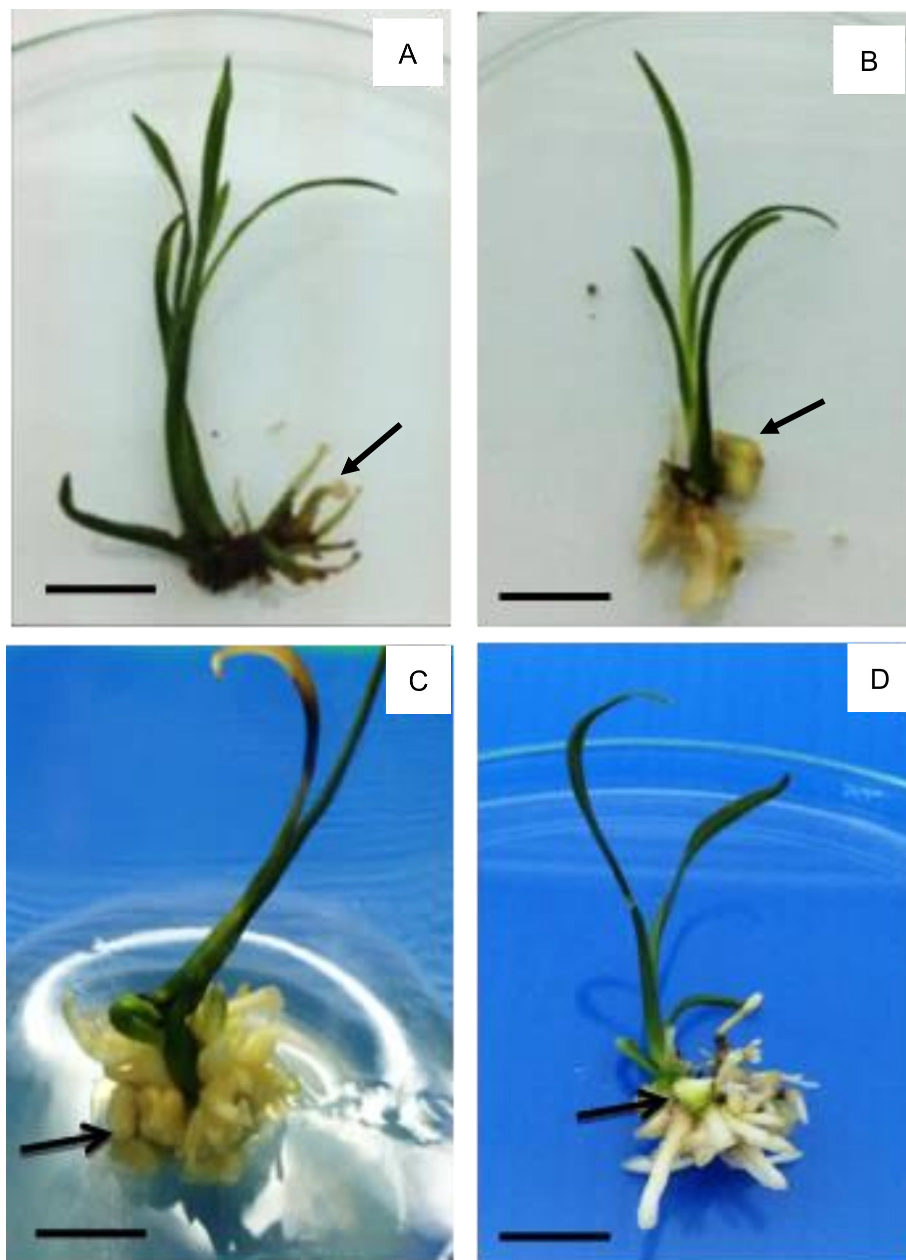

Figure 3.1 Directly induction of SE from root of oil palm plantlet after culture for two months. (A) Plantlet cultured on MS medium without growth regulators, showing normal roots (arrow). (B) Plantlet cultured on MS medium supplemented with 0.5 mg/L NAA, showing somatic embryo (arrow). (C) Plantlet cultured on OPCM medium supplemented with 0.5 mg/L NAA, showing somatic embryo (arrow). (D) Plantlet cultured on WPM medium supplemented with 1.0 mg/L NAA, showing somatic embryo (arrow) (bar = 1 cm).

Plant regeneration under in vitro conditions depends on various factors, such as, types of explant, basal medium, growth regulators, genotypes and culture conditions. Auxin, particularly 2,4-D or NAA are known to trigger the stimulation of pre-embryogenic determined cell to undergo cell division and then expression of embryogenesis in many plant species (Vankatachalam *et al*., 1999). The efficient regeneration of SE from different plant species depends strongly on the medium composition. OPCM medium is frequently used to induce somatic embryogenesis from culturing various explants of oil palm. Although OPCM medium has not been reported to use as culture medium for oil palm tissue culture before, we found that OPCM medium was the most suitable for this purpose in this present study. In contrast, MS and WPM medium gave only a low frequency of SE induction. The macroelements and microelements in OPCM medium were far different those in MS and WPM medium. The OPCM medium contains higher concentration of Cl- than WPM medium due to the presence of $CuCl_26H_2O$ and $CaCl_22H_2O$. Although Cl^- promotes root formation in the same manner as natural auxins, it may also play an important role in the growth and development of SEs in oil palm. The presence of NH_4NO_3 is thought to prevent the division of tissue in many woody plant species, such as poplar (Qiao *et al*., 1998). In the present study, we found that higher induction of SEs in OPCM medium might be due to an appropriate ratio of NH_4^+ to NO_3^-. The ratio of those chemicals was 4.1 : 3.8 which was higher concentration of NH_4^+ than that present in MS and WPM (Table 3.1). In the order to increase the nitrogen concentration in the medium, initially, MS medium, which contains nitrogen four times higher than that of WPM (1,650 mg/L and 400 mg/L) (Table 3.1) was selected to use for plant tissue culture. However, MS was reported to be less effective than WPM in the micropropagation of Bluecrop (Wolfe *et al*., 1983). Therefore, OPCM was used in order to optimize nitrogen quantity (between MS and WPM) for maximum growth and development of oil palm culture. In the present study, the average number of SEs and frequency of SE formation on MS medium supplemented with various concentrations of

dicamba was lower than that obtained on the other media. OPCM was the best among three culture media tested in SE induction and frequency of SE formation (Table 3.2). The results obtained in this present study was contradict to those of Bluecrop which WPM medium gave the best results (Wolfe *et al*., 1983). Another explanation may be a deficiency of microelements, since WPM contains neither cobalt nor iodine. MS often produces hyperhydric shoots because it is rich in ammonium ion as reported by Paques *et al*. (1995). Thus, to avoid generating hyperhydric shoots OPCM would be preferable to MS.

In the present study, SEs obtained from vitro shoot- derived roots on MS, WPM or OPCM medium supplemented with different concentrations of dicamba, 2,4-D or NAA were significant difference. OPCM supplemented with different concentrations of NAA showed the best response in term of frequency of SE formation and number of SEs, followed by of MS or WPM medium with dicamba or 2,4-D. Generally, 2,4-D is the most commonly used as auxin for the induction of SE in tissue culture of oil palm But in this study, contrary results was found. NAA showed the more potential in induction of SEs than 2,4-D. NAA was also reported to promote plantlet elongation significantly different in comparison with dicamba and 2,4-D (Vankatech *et al*., 2009). However, the other kinds of auxin did not promote plantlet growth. In many cases, embryogenesis is reported to occur into many stages, such as the induction of cells which undergo division to form embryogenic mass or proembryonic tissue in the presence of high concentration of 2,4-D and the development of embryogenic mass into somatic embryos in the absence, or presense of a low concentration of 2,4-D (Fujimura and Komamine, 1980). For germination of SEs types of auxin must be chanced or replaced by other PGRs. Vankatachalam *et al*. (1999) observed a higher frequency of somatic embryo germination in groundnut in the present of 8.88 μM BA and 0.26 μM NAA. Lehminger-Mertens and Jacobse (1989) have reported germination of somatic embryo derived from protoplast of pea on MS medium supplemented with 1.5-2.9 μM GA_3.

Histological analysis

Histological analysis of yellow to green SEs differentiated directly on vitro shoot-derived roots either singly or in groups after 1-2 months of culture revealed that most SEs developed in the proximal region of a single root. Development of each SEs was not synchronous. SEs appeared to differentiate from epidermal cells and a few parenchymatous cells. Parenchyma cells had large nucleus, dense cytoplasm with a broad basal area in contact to maternal tissue. They underwent rapid cell division to form proembryonic mass (PEM) and finally developed into somatic embryos through somatic embryogenesis (Figure 3.2C). The longitudinal root tip section from PGR-free MS medium showed normal structure with root cap, calyptrogens (actively dividing root cap cells), quiescent center and procambium (Figure 3.2A). Longitudinal section of SE showed the characterization of parenchymatous linkage between root and SE. All SEs had vascular connection from shoot apex to root zone or radicle (Figure 3.2C). However, some roots were not initiated into SEs (Figure 3.2B). Histological analysis reveals cells with embryogenic characteristics, such cells were usually found as niches of sub epidermal and epidermal tissue. In coconut somatic embryogenesis, subepidermal embryogenic cells also had a dense cytoplasm (Saenz *et al*., 2006).

Similarly, oil palm somatic embryos also appeared with a broad basal area in contact with maternal tissue, but without vascular connection with the maternal tissue, and histological analyses showed no relation with cell division during the initial development. All these characteristics suggest the single cell origin of the somatic embryos (Fernando *et al*., 2003). However plantlets have been regenerated mostly from somatic embryos with a multicellular origin (Pierik, 1990). This might be due to the lower concentrations of macronutrients containing OPCM and WPM than the MS media, in particular Ca and N (Table 3.1). The composition of the culture media has an important influence on the morphogenic response as demonstrated in the numerous investigation gathered by

Pierik (1990). The morphogenic response of a determined tissue is associated with the mineral nutrition that is applied. It is even possible to eliminate growth regulators by modifying the mineral composition of the culture medium (Ramage and Williams, 2002).

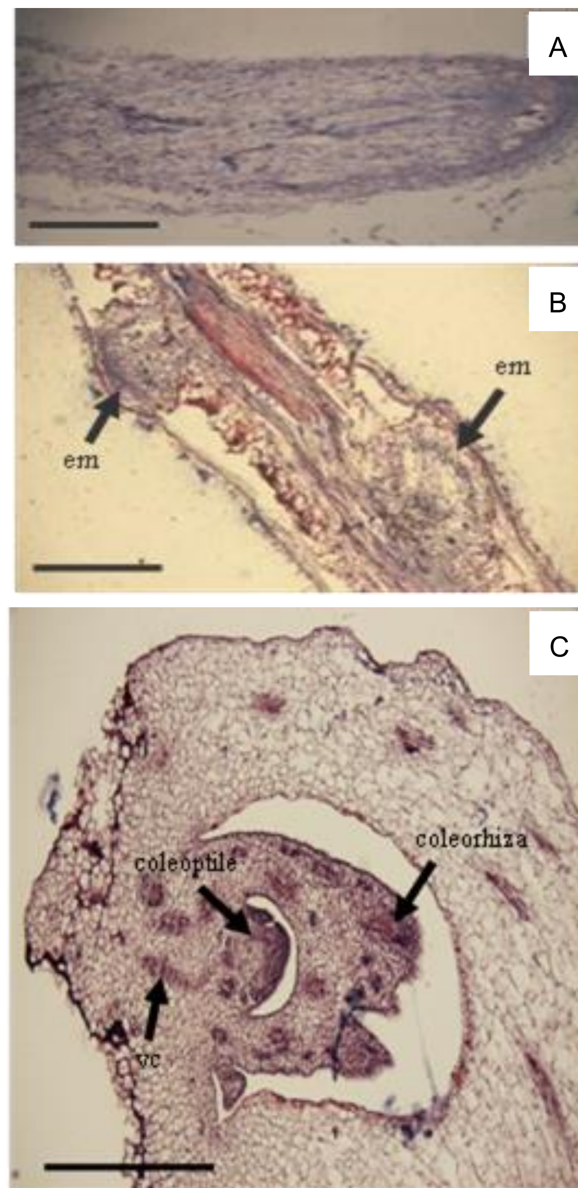

Figure 3.2 Histological studies of root and SE of oil palm after 2 months of culture. (A) Longitudinal section of root from PGR-free MS medium without somatic embryos (bar = 501.53 µm) showing normal root tip with 1; procambium, 2; quiescent center, 3; live root cap cells, 4; root cap. (B) Longitudinal section of root bearing somatic embryos em = embryonic mass (bar = 501.53 µm). (C) Longitudinal section of somatic embryo (bar = 800 µm) vc = vascular cambium.

CHAPTER IV

Proliferation of Oil Palm Embryogenic Callus and Conversion of Somatic Embryo into Plantlets:

Effect of Dicamba on Induction of Somatic Embryo and Plantlet Regeneration

Introduction

Plant tissue culture technique can apply for plant breeding in order to overcome some limitations and clonal propagation of oil palm (Aberlenc-Bertossi *et al*., 1999; Rajesh *et al*., 2003). Plant regeneration of oil palm through *in vitro* culture has been reported by several researchers (Te-chato and Muangkaewngam, 1992; Te-chato, 1998a; Te-chato, 1998b; Chehmalee and Te-chato, 2008). A reliable and efficient procedure for *in vitro* propagation of elite clones could significantly increase oil palm yields. Earlier studies from Crop Biotechnology laboratory, department of Plant Science, faculty of Natural Resources were based on regeneration from leaf explants using dicamba (Promchan and Te-chato, 2007). Dicamba has been reported to be an effective auxin for increasing a large number of SEs (Te-chato *et al*., 2003a). Te-chato (2001) reported that dicamba was superior to other auxins whereas NAA gave the poorest result in callus formation. Low concentration of dicamba at 2.5 mg/L in culture medium has been reported to be effective for primary callus induction from both zygotic embryos and young leaves (Te-chato *et al*., 2003a; Chehmalee and Te-chato, 2008). Moreover, dicamba has been reported to be an effective auxin for both shortening time period for callus induction and increasing a large number of somatic embryo (Te-chato *et al*., 2004). The objective of the present study was to develop culture medium for somatic embryo induction and plantlet regeneration from embryogenic callus of oil palm.

Materials and methods

Plant materials

Embryogenic callus of oil palm of breeding material from D×P crosses was provided by Golden Tenera Company cultured on MS medium supplemented with 1 mg/L dicamba, 3% sucrose and 200 mg/L ascorbic acid. The medium was solidified with 0.75% agar, adjusted to 5.7 prior to autoclaving at 1.05 kg/cm^2, 121 ^oC for 15 min. Cultures were maintained at 15 µmol/m^2/s (14 h photoperiod) at a temperature of 28±1 °C and sub-cultured after one month of incubation.

Effect of different concentrations of dicamba on somatic embryo induction.

Embryogenic callus at 0.1 gFW were cultured on OPCM medium supplemented with 0, 0.1, 0.3, 0.5 0.7, 0.9 and 1 mg/L dicamba, 3% sucrose and 200 mg/L ascorbic acid. The medium's pH was adjusted to 5.7 with 0.1 N HCl (hydrochloric acid) or KOH (potassium hydroxide) before adding 0.75% agar and autoclaved at 1.05 kg/cm^2, at 121 ºC for 15 min. The cultures were maintained at 28±1 ^oC under fluorescent lamps at 15 µmol/m^2/s for a 14 h photoperiod. There were 4 replicates each containing 5 testtubes per replicate. After being cultured for 3 months fresh weigh and characteristics of embryogenic callus were recorded and statistically compared. The data were statistically analyzed using CRD and the mean values among the treatments were separated by DMRT.

Effect of culture media on plant regeneration.

SSEs together with haustorium-staged embryos induced on sorbitol containing medium were transferred to different culture media (PGR-free MS medium, MS medium supplemented with 0.5 mg/L NAA or OPCM supplemented with 0.5 mg/L NAA). All culture media were supplemented with 3% sucrose, 200 mg/L ascorbic acid, solidified with 0.75% agar, adjusted to pH 5.7 before autoclaving and maintained at 28±1°C under 14 h photoperiod, 25 µmol/m^2/s. There were 4 replicates each containing 5 test tube per

replicate. After 3 months of culture (observed at monthly intervals) shoot, root and plantlet number were recorded and statistically compared. The data were statistically analyzed using CRD and the mean values among the treatments were separated by DMRT.

Results and Discussion

Effect of different concentrations of dicamba on somatic embryo induction.

After transfer EC initiated from zygotic embryo of oil palm on 2.5 mg/L dicamba containing MS medium for 3 months to OPCM medium with various concentrations of dicamba the medium with 0.1 mg/L dicamba gave the best response on EC proliferation at 0.33 gFW followed by dicamba at concentration of 0.3, 0.5, 0, 0.7, 0.9 and 1 mg/L, respectively. The highest percentage of SE formation was obtained from 0.1 mg/L dicamba containing OPCM at 30, significant difference with another concentration (Table 4.1, Figure 4.1). Dicamba was found to be the best auxin for *in vitro* mass propagation of both seedling and mature oil palm explant (Te-chato *et al*., 2003a). In additions, embryoids developed on medium containing 0.1 mg/L dicamba was found to be superior in inducing early stage of embryoid subsequent to further development of mature or haustorium embryoid (Te-chato, 1998a). Some authors reported that low concentration of dicamba promoted somatic embryogenesis from immature inflorescence (Steinmacher *et al*., 2007). Similar result was obtained in callus culture of *Areca catecha*. Decrease in concentration of dicamba stimulated proliferation rate of EC and also promoted a large number of embryoid formation of North American Grass (Wang *et al*., 2006).

Table 4.1 Effect of dicamba concentration containing OPCM medium on embryogenic callus multiplication and percentage of somatic embryo after culture for 3 months.

Conc. of dicamba (mg/L)	Fresh weight (g)	Somatic embryo formation (%)
0	0.28^{ab}	3.33^{c}
0.1	0.33^{a}	30.00^{a}
0.3	0.30^{ab}	16.67^{abc}
0.5	0.30^{ab}	5.00^{bc}
0.7	0.26^{b}	1.67^{c}
0.9	0.26^{b}	1.67^{c}
1.0	0.26^{b}	25.00^{ab}
F-test	*	*
C.V. (%)	10.53	44.99

* : significant difference at $P \leq 0.05$

Mean values followed by the same letter within each column are not significantly different according to DMRT

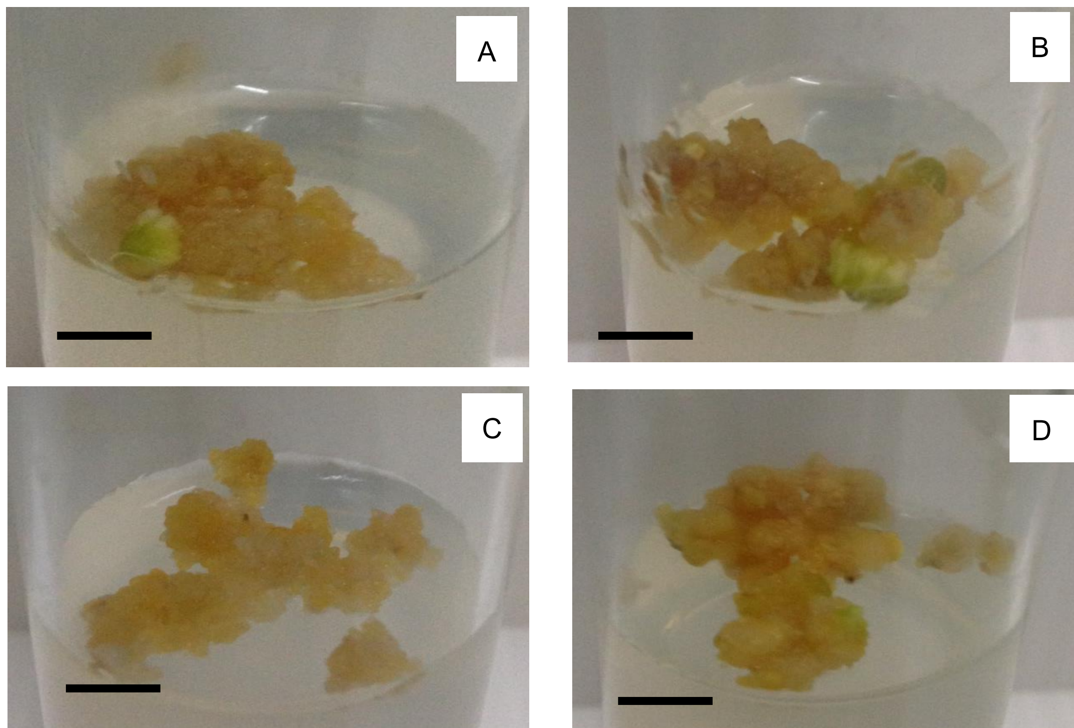

Figure 4.1 Embryogenic callus obtained from culture primary callus on OPCM medium. (A) without dicamba, (B) with 0.1 mg/L dicamba, (C) 0.7 mg/L dicamba and (D) 1 mg/L dicamba after culture for 3 months (bar = 0.5 cm).

Germination of SSEs

Increasing in period for maintaining SSEs on MS supplemented with 0.2 M sorbitol before transferring to three media, PGR-free MS medium, MS with 0.5 mg/L NAA and OPCM with 0.5 mg/L NAA promoted the different responses after 3 months of culture. PGR-free MS medium gave the highest result in shoot number at 10.4 shoots, root number at 8.20 roots and plantlet number at 7.20 plants (Table 4.2, Figure 4.2). Whereas, SSEs cultured on MS or OPCM medium supplemented with 0.5 mg/L NAA gave the average of shoot at 9.60 and 4.00 shoots and average number of root at 3.60 and 4.20 roots, respectively (Table 4.2, Figure 4.3). The different response might be related to the hydrolysis of food reserve in the endosperm and the mobilization of nutrient required for embryo germination like the report

of Sarasan (2005). Furthermore, this medium gave two to threefold higher root elongation rate than other media. It is likely that root induction may have occurred due to a high content of available nutrients and vitamins in the MN_6 medium, which would mediate synchronized development of shoots and roots from plantlets. Similar works reported significant improvements in plant regeneration with the increased levels of some nutrients in media in kodo and finger millets (Kothari-Chajer *et al*., 2008) The use of SSEs could provide an efficient solution to the problems limiting plant regeneration in oil palm like those reports in banana cultivars (Khalil *et al*., 2002). This also suggested that SSEs are probably of unicellular origin, making them an excellent candidate for genetic transformation since the potential for production of chimeric plants is low.

Table 4.2 Effects of MS or OPCM medium supplemented with 0.5 mg/L NAA on the development of SSEs after culture for 3 months.

Culture media	Shoot number (shoot)	Root number (root)	Plantlet number (plant)
MS-free	10.4±0.97	8.20±1.39	7.20±0.97
MS+0.5 mg/L NAA	9.60±0.97	3.60±0.87	3.40±0.75
OPCM+0.5 mg/L NAA	4.00±0.94	4.20±0.37	2.80±0.73
F-test	ns	ns	ns
C.V. (%)	27.10	40.79	41.28

ns: not significant difference

Figure 4.2 Development of SSEs on different culture media, (A) PGR-free MS, (B) MS + 0.5 mg/L NAA, (C) OPCM + 0.5 mg/L NAA after 3 months of culture (bar = 0.5 cm).

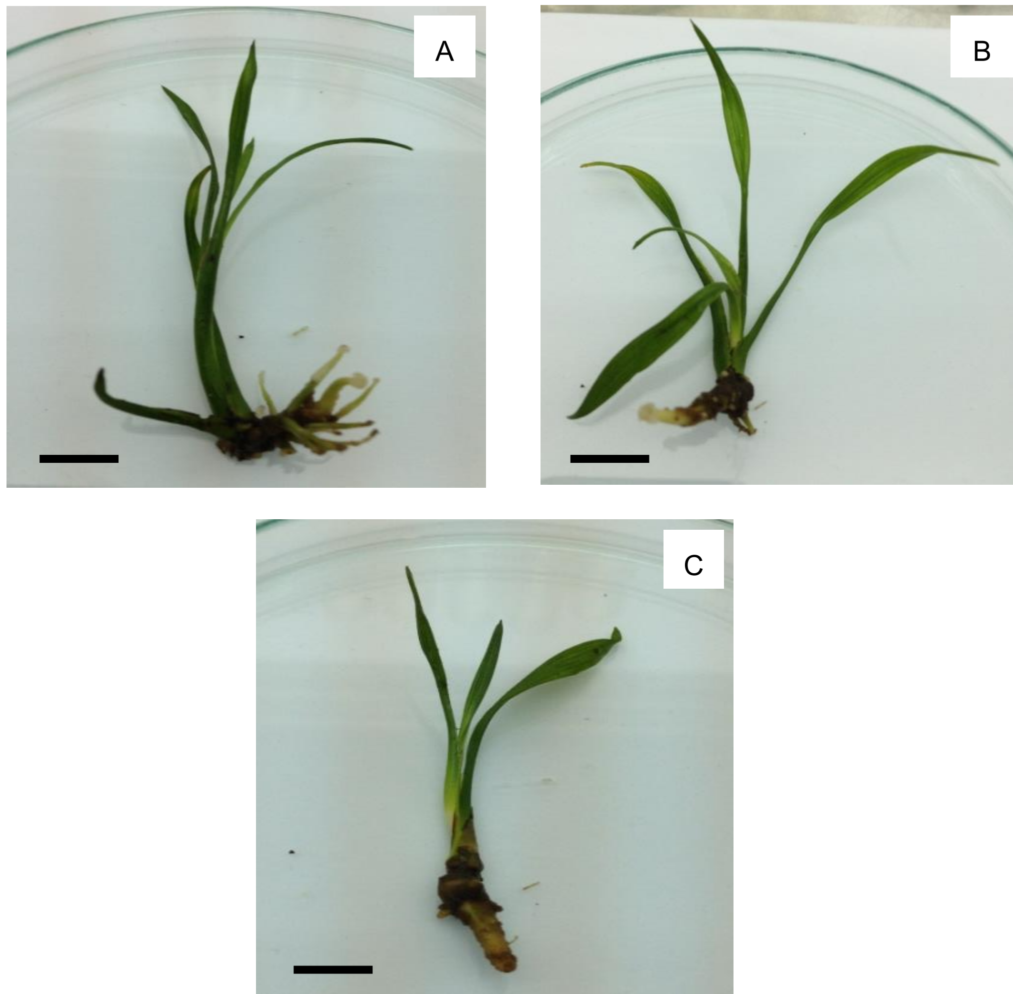

Figure 4.3 Germination of oil palm seedling on (A) PGR-free MS medium, (B) MS + 0.5 mg/L NAA, (C) OPCM + 0.5 mg/L NAA after 3 months of culture (bar = 0.5 cm).

CHAPTER V

Assessment of Genetic Variability:

Assessment of Somaclonal Variation of EC-derived Plantlets of Oil Palm from TIS Culture by SSR Marker

Introduction

Genetic instability of oil palm somaclone obtained from *in vitro* culture should be assessed at early stage of development, which would reduce time, cost and resources required for misplanting. Recently, molecular marker techniques based on the PCR has been one of the most commonly used for characterization of genetic variability, genotype identification, genetic instability analyses and selection and breeding purposes (Tingey and Tufo, 1993). Among those techniques SSR was proved to be very commonly used in identification hybridity of oil palm F1 hybrid (Thawaro and Te-chato, 2010). To make sure that long-term culture of embryogenic callus of elite oil palm clone can produce uniform plantlets MAS is necessary. The highly polymorphic nature of SSR is of particular importance for oil palm, as the breeding palms often involve narrow gene pools. Additionally, it was demonstrated that the SSR markers can be used for clonal identification, monitoring line uniformity between and within clones and detecting culture mix-up in oil palm (Singh *et al*., 2007).

Molecular markers have been reported to assess genetic variability in cultivated clones, wild type, clonal uniformity and evaluation of genetic diversity in poplar clones (Roy *et al*., 2004). SSR technique was reported to be successful in identification and characterization of somaclonal variation of plants such as oil palm (Inpuay *et al*., 2012), poplar (Rahman and Rajora, 2001) and guava (Rai *et al*., 2012). The objective of the present study was to assess genetic variability of *in vitro* embryogenic callus from TIS culture by SSR marker.

Materials and methods

Plant materials and culture conditions

The EC cultured EC medium which was MS medium supplemented with 1 mg/L dicamba, 3% sucrose and 200 mg/L ascorbic acid for 1 month was transferred to EC proliferation medium (MS supplemented with 1 mg/L dicamba, 3.75% sucrose and 200 mg/L ascorbic acid) in TIS system, at a temporary immersion cycle of 3 min with 150 mL EC proliferation medium every 8 h. The cultures were maintained at 28±1 ^{o}C under fluorescent lamps at 15 µmol/m^{2}/s for a 14 h photoperiod. EC was collected and used for genetic variability assessment by SSR.

Verification with SSR technique

EC (0.5 gFW) derived from EC proliferation medium with 3.75% sucrose in TIS systemwas collected and DNA isolation carried out following the method described by Te-chato (2000). For SSR analysis of genomic DNA 9 microsatellite loci amplified primers (EgCIR0008, EgCIR0243, EgCIR0337, EgCIR0409, EgCIR0446, EgCIR0465, EgCIR0781, EgCIR0905 and EgCIR1172) from oil palms were used. The primers were obtained from Operon Tech. (California, USA). Amplification of genomic DNA was performed according to the protocol of Billotte *et al*. (2005). Each amplification mixture of 10 µl contained 1 µl of 2.5 mM $MgCl_2$, 1 µl of 10x *Taq* buffer, 2 µl of 1mM dNTP, 0.5 µl of 0.3 mM primer, 0.1 µl of *Taq* polymerase, 4.1 µl of dH_2O 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 ºC for 60 s, 35 cycles of 94 ºC for 30 s, 52 ºC for 60 s, and 72 ºC for 120 s and a final elongation step at 72 ºC 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 ºC for 5 min. The products were separated on 6% (W/V) denaturing

polyacrylamide gels. Silver staining was conducted according to the protocol of Benbouza *et al*. (2006).

Results and Discussion

Verification with SSR technique

All primers could amplify and provide monomorphic patterns of the DNA from both solid medium and TIS. The number of amplified banding DNA varied among 9 primers with an average bands of 5.44 bands. The size of amplified banding DNA ranged from 100 to 250 bps. The all of monomorphic SSR loci generated at 100% (Table 5.1, Figure 5.1, 5.2). The results obtained from this study suggest that there is no somaclonal variation or genetic instability occurs in EC derived from both solidified medium and TIS. The basic objective of micropropagation is to produce the true-to-type plants, therefore, it is important of certify and retain the fidelity of produced regenerants. SSR analyse have been used to assess the genetic fidelity of micropropagated regenarants. This molecular technique by pass the reliance on diagnostic morphogical and phytochemical traits that take time to collect in mass propagation system and assure the fidelity of micropropagated plants (Nayak *et al*., 2003). In oil palm, SSR analysis was used to examine genotype identification (Billotte *et al*., 2001), genetic mapping (Billotte *et al*., 2005) and population genetic (Singh *et al*., 2007). Using SSR technique, various researchers have reported the absence of genetic variation in oil palm. Thawaro (2009) reported that SSR analysis of regenerated plantlets with EgCIR1772 primer provided clear DNA patterns and monomorphism band. The result revealed that there was no somaclonal variations detected by this technique. Followed by Sanputawong (2010) also reported that SSR marker with primer EgCIR0008 gave a uniformity of plantlets derived from immature zygotic embryo culture. The absence of any sign of somaclonal variation from this study was found using SSR marker.

Table 5.1 Types and base sequences of primer SSR used for amplifying genomic DNA.

Primer	Sequence (5'-3')	Annealing Temperature (ºC)	Allele size	Amplified fragment	Monomorphic (%)
EgCIR0008	(F) CGGAAAGAGGGAAGATG	52	105-150	11	100
	(R) ACCTTGATGATTGATGTGA				
EgCIR0243	(F) TGGAACTCCTATTTTACTGA	52	248-270	2	100
	(R) GCCTCGTAATCCTTGTCA				
EgCIR0337	(F) GTCTGCTAAAACATCAACTG	52	145-189	4	100
	(R) GAGGAGGAGGGGAACGATAA				
EgCIR0409	(F) AGGGAATTGGAAGAAAAGAAAG	58	252-298	4	100
	(R) TCCTGAGCTGGGGTGGTC				
EgCIR0446	(F) CCCCTTCGAATCCACTAT	52	202-275	3	100
	(R) CAAATCCGACAAATCAAC				
EgCIR0465	(F) TCCCCCACGACCCATTC	52	125-197	6	100
	(R) GGCAGGAGAGGCAGCATTC				
EgCIR0781	(F) CCCCTCCCTACCACGTTCCA	52	207-288	5	100
	(R) TGTTTGCTGTTGCTCTTTGATTTTC				
EgCIR0905	(F) CACCACATGAAGCAAGCAGT	52	231-284	9	100
	(R) CCTACCACAACCCCAGTCTC				
EgCIR1772	(F) ACCTTGTATTAGTTTGTCCA	52	166-198	5	100
	(R) CTTCCATTGTCTCATTATTCTCTTA				
Average				5.44	100

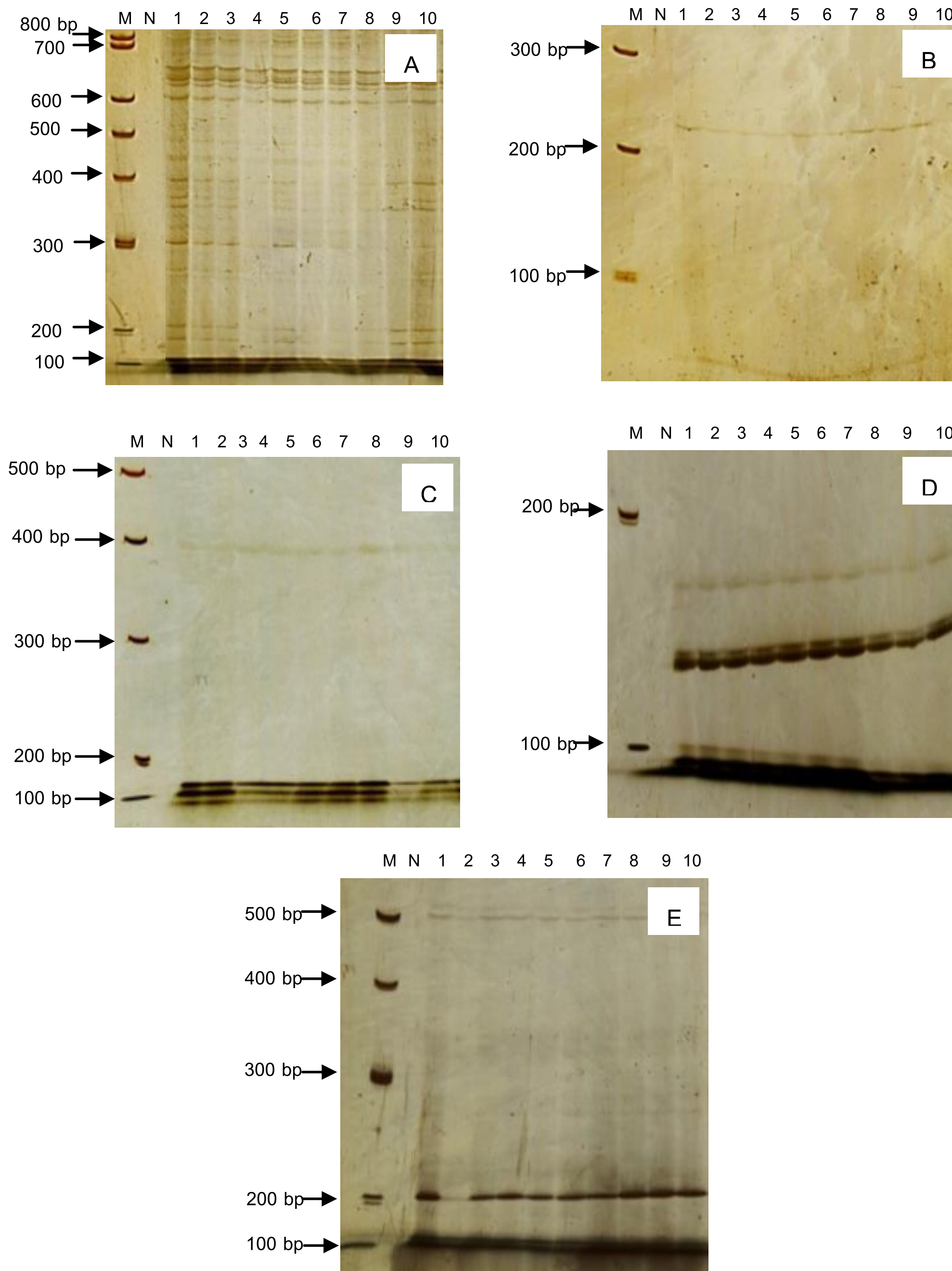

Figure 5.1 Profiles of genomic DNA isolated from EC of oil palm and amplified by PCR-based SSR marker. The amplification products were compared on the basis of standard molecular marker. Lane M: DNA ladder, Lane N: Negative control, Lanes 1-5: EC derived from solid medium and Lanes 6-10: EC derived from TIS, obtained with primers (A) EgCIR0008, (B) EgCIR0243, (C) EgCIR0337, (D) EgCIR0409 and (E) EgCIR0446.

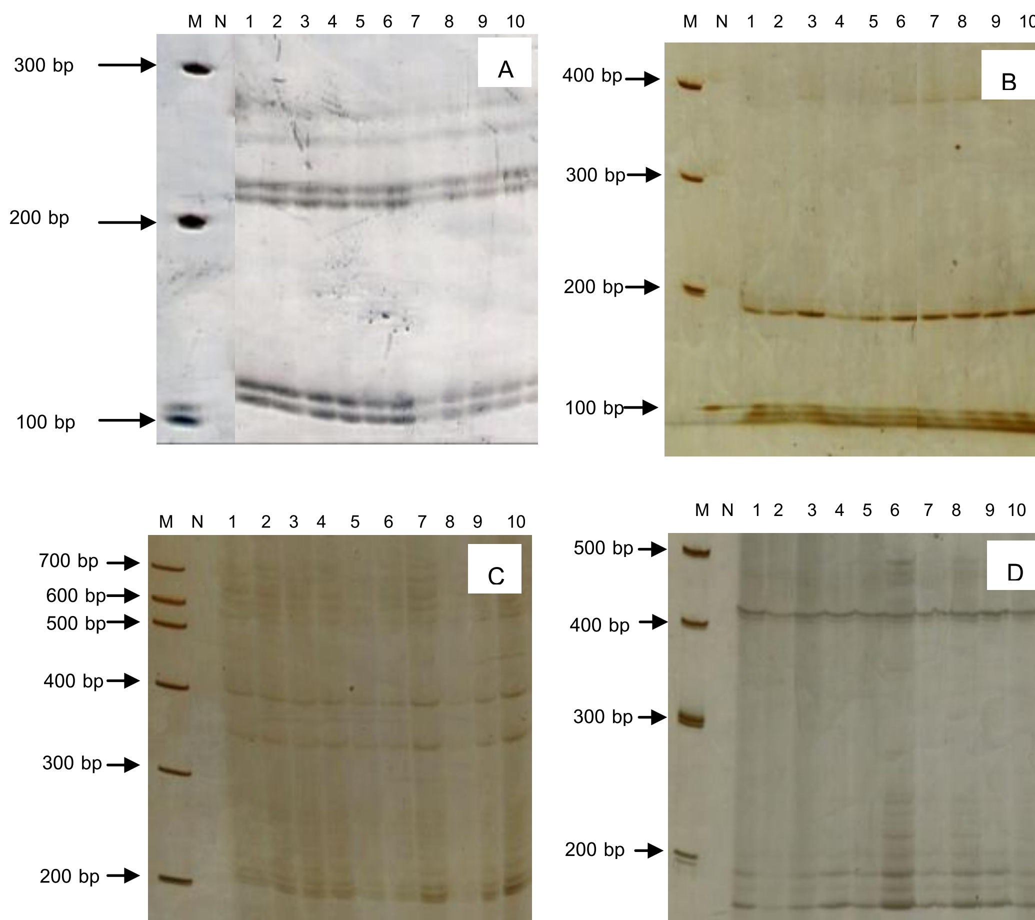

Figure 5.2 Profiles of genomic DNA isolated from EC of oil palm and amplified by PCR-based SSR marker . The amplification products were compared on the basis of standard molecular marker. Lane M: DNA ladder, Lane N: Negative control, Lanes 1-5: EC derived from solid medium and Lanes 6-10: EC derived from TIS, obtained with primers (A) EgCIR0465, (B) EgCIR0781, (C) EgCIR0905 and (D) EgCIR1772.

CHAPTER VI

Concluding Remarks

Concluding Remarks

Embryogenic callus proliferation

The most suitable system for EC was obtained in TIS at temporary immersion for 3 min followed by drying period for 8 h with volume of medium at 150 mL in EC medium (MS medium supplemented with 0.1 mg/L dicamba, 200 mg/L ascorbic acid and 3.75% sucrose after culture for 1 month. That result in the highest growth ratio of EC proliferation was 11.19, This was significant different from other culture systems

Direct somatic embryogenesis

The high frequency direct somatic embryogenesis of oil palm by culturing plantlet on OPCM medium supplemented with 0.5 mg/L NAA and 200 mg/L ascorbic acid. Roots of vitro-plantlets gave the highest number of SEs at 1.2 SEs and percentage of SE formation at 80. Histological study revealed that SE appeared to differentiate from epidermal cells and few parenchymatous cells. Parenchyma cells appeared with a broad basal area in contact to maternal tissue, and without vascular connection with the maternal tissue. This morphogenetic pathway allowed the development of a protocol suitable for direct somatic embryo formation from roots of *in vitro*-seedlings of oil palm.

Indirect somatic embryogenesis

For proliferation of EC and formation of SE, OPCM medium supplemented with 0.1 mg/L dicamba, 200 mg/L ascorbic acid, 3% sucrose and 0.75% agar gave fresh weight of EC at 0.33 g. SEs were continuously formed from EC on the same medium promoted SE formation at 30% after 3 months of culture, significant on the fresh weight of EC and SE formation. Upon transferring SSE to MS medium without plant growth regulator with 3% sucrose, 200 mg/L ascorbic acid for 3 months, the highest number of shoots at 10.4

shoots/explant, number of root at 8.20 roots/explant and complete plantlets at 7.20 plantlets/explant were obtained.

Genetic variability

SSR marker revealed uniformity of somaclone obtained from culturing EC of oil palm in TIS culture for 3 min followed by drying period for 8 h, volume of medium at 150 mL per flask in EC medium after culture for 1 month. EC obtained by this procedure had the same profiles of DNA patterns as revealed by 9 primers of SSR marker. DNA patterns of EC showed no somaclonal variation or genetic variability occurs in EC derived from solidified medium and TIS.

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APPENDICES

Appendix A

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

Components	Volume (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
Thiamine HCl	0.100
Glycine	2.000
Sucrose	30,000.00
Agar	7,500.00
pH	5.7

Table 2 Composition of nutrition of Oil Palm Culture Medium (OPCM)

Components	Volume (mg/L)
Macro elements	
NH_4NO_3	1,025.000
KNO_3	950.000
KH_2PO_4	170.000
$CaCl_2.2H_2O$	268.000
$MgSO_4.7H_2O$	185
Micro elements	
KI	0.415
K_2SO_4	495.000
H_3BO_3	6.200
$MnSO_4.H_2O$	16.900
$ZnSO_4.7H_2O$	9.600
$CuSO_4.5H_2O$	3.138
$Na_2MoO_4.2H_2O$	0.250
$CoCl_2.6H_2O$	0.0125
$FeSO_4.7H_2O$	27.800
Na_2EDTA	37.300
Organic compounds	
Myo-inositol	100.000
Nicotinic acid	0.500
Pyridoxine HCl	0.500
ThiamineHCl	0.550
Glycine	2.000
Sucrose (g)	30.000
Agar (g)	7.500
pH 5.7	5.7

Table 3 Composition of nutrition of Woody Plant Medium (WPM)

Components	Volume (mg/L)
Macro elements	
NH_4NO_3	400.000
KNO_3	-
KH_2PO_4	170.000
$CaCl_2.2H_2O$	96.000
$MgSO_4.7H_2O$	-
Micro elements	
KI	-
K_2SO_4	990.000
H_3BO_3	6.200
$MnSO_4.H_2O$	16.900
$ZnSO_4.7H_2O$	8.600
$CuSO_4.5H_2O$	6.250
$Na_2MoO_4.2H_2O$	0.250
$CoCl_2.6H_2O$	-
$FeSO_4.7H_2O$	27.800
Na_2EDTA	37.300
Organic compounds	
Myo-inositol	100.000
Nicotinic acid	0.500
Pyridoxine HCl	0.500
ThiamineHCl	0.100
Glycine	2.000
Sucrose (g)	30.000
Agar (g)	7.500
pH 5.7	5.7

Appendix B

Preparation of buffer solution and reagents for molecular marker analysis

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) and 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 8.0)
- 200 μL of 0.25 M Na_2EDTA (pH 8.0)

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 Na_2EDTA (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 Na_2EDTA (pH 8.0)

Adjust the volume to 4 L with distilled water and sterilize by autoclaving.

Appendix C

Preparation of paraffin method and safranin and fast green staining

1. Paraffin method

The tissue must be fixed in FAA II, washed, dehydrated, infiltrated, embedded in paraplast before being sectioned. The steps of paraffin preparation are as follows:

- Collecting and fixing in fixative
- Dehydration
- Infiltration
- Embedding
- Microtome sectioning
- Mounting paraffin sections
- Deparaffinization
- Staining

1.1 Fixation

FAA (formalin, acetic acid, alcohol; 3:1:1) can be stored in dark and cool

- Drop samples into FAA II
- Apply vacuum (optimal)
- Incubate for 4-24 h
- Transfer to 50% alcohol
- Transfer to 70% alcohol for storage

1.2 Dehydration series (EtOH series)

Fixed samples are washed in 70% alcohol (2 times) and begin dehydration process use EtOH series from #5 or 6 to #12, 2 h/ step.

1.3 Infiltration and embedding

- Fixed samples (in #12) are transfer to solid paraplast 1 (P1)

- Then they are placed in paraffin oven 2 h
- Pour P1 to paraffin waste bag and fill up samples with molten paraplast 2 (P2), let melt in oven (58 ºC) for 2 h
- Replace with molten paraplast 3 (P3) and let melt again (overnight is recommended for the last step)
- Proceed with embedding (using paraffin embedding center)

1.4 Deparaffinization protocol

Before proceeding with staining, all slide must be deparaffinized and rehydrated. Placed the slides in coplin jar and performed the following wash;

- Xylene substitute I 3 min
- Xylene substitute II 3 min
- Absolute ethanol : Xylene substitute (1:1) 3 min
- Absolute ethano II 2 min
- Absolute ethano III 2 min
- 95% ethano II 2 min
- 95% ethano III 2 min
- 70% ethano II 2 min
- 70% ethano III 2 min

2. Safranin and fast green staining

2.1 Deparaffinization and safranin staining

- Deparaffinization (paraffin sections down to 70% alcohol)
- Stain in safranin for at least 18 h
- Wash out excess with water for a few moments

2.2 Microscopy observation

- Differentiate carefully in 50% alcohol containing 1-2 drops of HCl

2.3 Dehydration

- 95% alcohol + 0.5% picric acid 10 sec
- 95% alcohol + 4 drops of ammonium hydroxide/100 mL alcohol 10 sec
- Absolute alcohol 10 sec

2.4 Counterstaining with fast green

- Drop used clove oil fast green and pour back into dropping bottle
- Counterstain with fast green and pour counterstain back into dropping 10 sec
- Rinse off excess stain with used clove oil fast green
- Drop new clove oil and observe under microscope
- Clear in a mixture of new clove oil : Absolute alcohol : xylene substitute (1:1:1)
- Washing slide a few seconds in Absolute alcohol : xylene substitute (1:1)
- Two changes of xylene substitute
- Mount

PAPER 1

Effect of Dicamba on Induction of Somatic Embryo and Plant Regeneration of Oil Palm (*Elaeis guineensis* Jacq.)

PAPER 2

Direct Somatic Embryo Formation from Roots of *In Vitro*-Seedlings of Oil Palm

(*Elaeis guineensis* Jacq.)

PAPER 3

Proliferation of Embryogenic Callus of Oil Palm (*Elaeis guineensis* Jacq.) Using Different Culture Systems and Genetic Instability Assay by Simple Sequence Repeat (SSR) Technique

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