Screening and Detection of Hybrid Oil Palms by DNA Markers and Theirs Propagation

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ชื่อวิทยานิพนธ์	การคัดเลือกและตรวจสอบพันธุ์ปาล์มน้ำมันลูกผสมโดยเครื่องหมายดีเอ็นเอและการขยายพันธุ์
ผู้เขียน	นางสาวสุภาวดี ถาวโร
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บทคัดย่อ

คัพภะปาล์มน้ำมันลูกผสมจำนวน 6 คู่ผสม ได้แก่ คู่ผสม 366 (D) × 172 (P), 366 (D) × 72 (P), 366 (D) × 206 (P), 865 (D) × 206 (P), 110 (D) × 865 (P), 366 (D) × 777 (P) เพาะเลี้ยงบนอาหารสูตร Murashige and Skoog (MS) เติมชนิดและความเข้มข้นของออกซินต่างๆ พบว่าจีโนไทป์ ชนิด และความเข้มข้นของออกซินมีผลต่อการสร้างแคลลัสและการชักนำแคลลัสชนิดต่างๆ ทุกคู่ผสมสามารถชักนำแคลลัสได้บนอาหารเพาะเลี้ยงสูตร MS เติม 3,6-dichloro-o-anisic acid (ไดแคมบา) เข้มข้น 2.5 มิลลิกรัมต่อลิตร โดยคู่ผสมเบอร์ 58 ให้เปอร์เซ็นต์การสร้างแคลลัสแบบปุ่มปม (48.50) และจำนวนแคลลัสแบบปุ่มปมเฉลี่ย (18.78) สูงสุด เมื่อเพาะเลี้ยงบนอาหารสูตร MS เติมไดแคมบาเข้มข้น 2.5 มิลลิกรัมต่อลิตร แตกต่างกันทางสถิติอย่างมีนัยสำคัญกับคู่ผสมอื่นๆ เมื่อศึกษาลักษณะเนื้อเยื่อวิทยาของการเกิดแคลลัสแบบปุ่มปมที่เพาะเลี้ยงบนอาหารเติม 2,4-dichlorophenoxyacetic acid (2,4-D) มีจุดกำเนิดมาจากเซลล์บริเวณชั้นอิพิเดอร์มิส ในขณะที่อาหารเติมไดแคมบาให้จุดกำเนิดมาจากเซลล์บริเวณชั้นอิพิเดอร์มิสและวาสคิวลาร์แคมเบียม ส่วนอาหารเติม α-naphthalene acetic acid (NAA) ให้การสร้างแคลลัสคล้ายราก มีจุดกำเนิดรากและพัฒนาต่อไปเป็นราก คู่ผสมเบอร์ 58 ให้เปอร์เซ็นต์การสร้างเอ็มบริโอเจนิคแคลลัส (embryogenic callus: EC) (50.65) และจำนวน EC เฉลี่ย (9.14) สูงสุด แตกต่างกันทางสถิติอย่างมีนัยสำคัญกับคู่ผสมอื่นๆ หลังจากเพาะเลี้ยงเป็นเวลา 3 เดือน คู่ผสมเบอร์ 58 ให้เปอร์เซ็นต์การสร้างโซมาติกเอ็มบริโอระยะรูปกลม (globular stage: GS) (17.32) ระยะสร้างจาว (haustorium stage: HS) (7.92) จำนวน GS เฉลี่ย (13.38) และ จำนวน HS (4.00) เฉลี่ย สูงสุด แตกต่างกันทางสถิติอย่างมีนัยสำคัญกับคู่ผสมอื่นๆ หลังจากเพาะเลี้ยงบนอาหารสูตร MS เติมไดแคมบาเข้มข้น 1 มิลลิกรัมต่อลิตร ร่วมกับกรดแอสคอร์บิคเข้มข้น 200 มิลลิกรัมต่อลิตร เป็นเวลา 3 เดือน เมื่อย้ายเลี้ยงในอาหารสูตร MS เติมน้ำตาลซอร์บิทอลเข้มข้น 0.2 โมลาร์ ร่วมกับกรดแอสคอร์บิคเข้มข้น 200 มิลลิกรัมต่อลิตร พบว่า HS ของคู่ผสมเบอร์ 58 ให้การพัฒนาโซมาติกเอ็มบริโอชุดที่ 2 (secondary

somatic embryos: SSE) (36%) และให้จำนวน SSE เฉลี่ย (21.56) สูงสุด แตกต่างกันทางสถิติอย่างมีนัยสำคัญกับคู่ผสมอื่นๆ หลังจากเพาะเลี้ยงเป็นเวลา 4 เดือน และย้ายเลี้ยงกลุ่ม SSE ของทุกคู่ผสม บนอาหารที่ปราศจากสารควบคุมการเจริญเติบโตเป็นเวลา 3 เดือน พบว่า คู่ผสมเบอร์ 58 ให้เปอร์เซ็นต์การงอกเป็นพืชต้นใหม่ (38.57) สูงสุด แตกต่างกันทางสถิติอย่างมีนัยสำคัญกับคู่ผสมอื่นๆ

การตรวจสอบพันธุ์ปาล์มน้ำมันลูกผสมเทเนอราระหว่างพันธุ์พ่อพิสิเฟอร่า (เบอร์ 72, 110, 172, 206, 558 and 777) กับพันธุ์แม่ดูร่า (เบอร์ 366 และ 865) ด้วยเครื่องหมายอาร์เอพีดีและเอสเอสอาร์ นำดีเอ็นเอจากใบของต้นพันธุ์พ่อ-แม่ที่ปลูกในแปลงทดสอบ และคัพภะที่ได้รับการตัดแบ่งครึ่งมาตรวจสอบโดยเทคนิคอาร์เอพีดีและเอสเอสอาร์ จากการใช้ไพร์เมอร์อาร์เอพีดีจำนวน 7 ไพร์เมอร์ พบว่ามีเพียงไพร์เมอร์ OPT06 ให้แถบดีเอ็นเอได้ชัดเจนและสามารถตรวจสอบความเป็นลูกผสมของคู่ผสมเบอร์ 77 และ 119 โดยให้แถบดีเอ็นเอจำเพาะขนาด 650 และ 400 คู่เบส ตามลำดับ ส่วนการใช้ไพร์เมอร์เอสเอสอาร์จำนวน 9 ไพร์เมอร์ พบว่ามี 2 ไพร์เมอร์ที่ให้แถบดีเอ็นเอได้ชัดเจนและสามารถตรวจสอบลูกผสมได้ โดยไพร์เมอร์ EgCIR008 สามารถตรวจสอบลูกผสมเบอร์ 77, 118, 119 และ 137 ส่วนไพร์เมอร์ EgCIR1772 สามารถตรวจสอบลูกผสมเบอร์ 58 และ 130 การตรวจสอบความแปรปรวนทางพันธุกรรมของ GS และต้นกล้าปาล์มน้ำมันลูกผสมด้วยเทคนิคอาร์เอพีดี ไม่พบความแปรปรวนของ GS และต้นกล้าปาล์มน้ำมันลูกผสมเบอร์ 77 ส่วนการตรวจสอบความแปรปรวนทางพันธุกรรมของ GS และต้นกล้าปาล์มน้ำมันลูกผสมด้วยเทคนิคเอสเอสอาร์ ไม่พบความแปรปรวนของ GS และต้นกล้าปาล์มน้ำมันทุกคู่ผสม

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ABSTRACT

Mature zygotic embryos (MZEs) of six genotypes of *Elaeis quineensis* Jac DxP; 366 (D) × 172 (P), 366 (D) × 72 (P), 366 (D) × 206 (P), 865 (D) × 206 (P), 110 (D) × 865 (P), 366 (D) × 777 (P) were cultured on Murashige and Skoog (MS) medium plus various kinds and concentrations of auxins. The effects of genotypes and auxins on callus forming percentage and type of callus were studied. The best result in callus formation was achieved on MS medium supplemented with 2.5 mg/l 3,6-dichloro-o-anisic acid (dicamba) in all genotypes. However, types of callus were quite different. Cross #58 gave the highest percentage of nodular callus formation (48.50) and average number of nodular callus (18.78) when cultured on 2.50 mg/l dicamba containing medium, significant difference to other genotypes. Histological study revealed that origin of nodular callus obtained from 2,4-D containing medium arose from only epidermal cell layer while dicamba induced from both epidermal and vascular cells. NAA containing medium provided root like calluses which developed root primodia subsequent to root formation. For embryogenic callus (EC) formation, cross #58 gave the result both in percentage of EC (50.65) and average number of EC (9.14), significantly different to other genotypes after 3 months of culture. In case of somatic embryo (SE) formation, cross #58 was identified as the best genotype for producing highest percentage of SE at globular stage (GS) (17.32) and haustorium stage (HS) (7.92) when cultured on MS medium supplemented with 1 mg/l dicamba and 200 mg/l ascorbic acid after 3 months of culture. In addition, SE in term of formation at GS and HS were achieved at 13.38 and 4.00 embryos/EC, respectively. In case of Secondary somatic embryos (SSEs) formation, it

formed from the basal part of HS after 4 months of transfer onto MS supplemented with 0.2 M sorbitol and 200 mg/l ascorbic acid known as stress medium. Cross #58 gave the result in percentage of GS (17.32), HS (7.92), average number of GS (13.38) and HS (4.00), significantly different to other genotypes after 3 months of culture.Some of those SSEs developed into mature embryos with green shoots. Germination of shoots and complete plantlets were observed just after one month of transfer onto PGR-free MS medium.

Hybrid verifications within the F1 populations (Tenera) between Pisifera (72, 110, 172, 206, 558 and 777) and Dura (366 and 865) were detected by random amplified polymorphic DNA (RAPD) and simple sequence repeat (SSR) analysis. Firstly, DNA from half mature zygotic embryos (HMZEs) of six combinations of hybrid were isolated and detected by RAPD using 7 RAPD primers and 9 SSR primers. From all random primers tested, they could amplify parental DNA. Primer OPT06 provided clearly DNA pattern and could be used for hybrid verification of the cross #77 and cross #119. DNA patterns among cross #77 and cross #119 had specific fragment at 650 and 400 bps, respectively. In the other analysis, we were successfully developed the verification technique of oil palm hybrid by SSR analysis using HMZEs-cultured. Two SSR primers were proved to be suitable for verification of six different hybrid combinations. For EgCIR008 primer, it could access verify more than one cross combination: cross #77, cross #118, cross #119 and cross#137 and had a wide range of verification the highest information capacity. In case of primer EgCIR1772, it was able to identify the hybrid from two crosses; cross #58 and cross #130.

The assessment of the genetic stability of *in vitro* derived clones at an early stage of development is considered to be very useful and is an essential step in this study. RAPD analysis was used to assess genetic stability in somatic embryo at GS and micropropagted plantlets of only cross #77. Somaclonal variation was not observed among individual somatic embryo at both levels. For SSR analysis of SE at GS, uniformity assessment was obtained in all genotypes. The results of this present study found that somaclonal variation were not detected. Therefore, efficiency of two DNA makers in hybrid verification and assessment of somaclonal variation were satisfied.

Contents

Contents (Continued)

List of tables

List of illustrations

List of illustrations (Continued)

List of illustrations (Continued)

List of illustrations (Continued)

List of illustrations (Continued)

List of abbreviations and symbols

ABA	=	Abscisic acid
AFLP	=	Amplified Fragment Length Polymorphism
BA	=	6-benzyladenine
bp	=	Base pair
CRD	=	completely random design
CTAB	=	Hexadecyltrimethylammonium bromide
2,4-D	=	2,4-Dichlorophenoxyacetic acid
Di	=	Dicamba
DMRT	=	Duncan's multiple range test
DNA	=	Deoxyribonucleic acid
dNTP	=	Deoxynucleotide triphosphate
EDTA	=	Ethylenediaminetetraacetic acid
Ep	=	Epidermis
FAA	=	Formalin: glacial acetic acid: 70 % ethanol
FELDA	=	Federal Land Development Authority
GA_3	=	Gibberellic acid
HCl	=	Hydrochloric acid
HE	=	Haustorium embryo
HL	=	Hoagland (medium)
M	=	Molar
M_1	=	DNA ladder 1000 bps
M_2	=	DNA ladder 100 bps
MS	=	Murashige and Skoog (medium)
NAA	=	α- naphthalene acetic acid
$NaCl_2$	=	Sodium chloride
Na_2EDTA	=	Sodium ethylenediaminetetraacetate
PCR	=	Polymarese chain reaction

PVP	=	Polyvinyl pyrrolidone
RAPD	=	Random amplified polomorphic DNA
RFLP	=	Restriction Fragment Length Polymorphism
SD	=	Standard deviation
SDS	=	Sodium dodecyl sulfate
SE	=	Somatic embryo
SH	=	Schenk and Hildebrandt (medium)
SPSS	=	Statistical Package for Social Science
SSE	=	Secondary somatic embryo
SSR	=	Simple Sequence Repeats
TDZ	=	Thidiazuron
TE	=	Tris EDTA
TAE	=	Tris-acetic acid-disodium ethylenediaminetetraacetic acid
TBE	=	Tris-boric acid- disodium ethylenediaminetetraacetic acid
Tris	=	Tris (hydroxymethyl) aminomethane
VB	=	Procambium or vascular cambium
UPGMA	=	Unweighted Pair-Group Method Using Arithmetic Average
ZR	=	Zeatin riboside

CHAPTER 1

INTRODUCTION

CHAPTER 1

INTRODUCTION

Background

Oil palm (*Elaeis guineensis* Jacq.) is a diploid monocotyledon belonging to the family Arecaceae. It is an economically important source of vegetable oil, the most traded vegetable oil in the international market, and increasingly used in the food industry (Corley and Tinker, 2003). In Thailand, the Thai government aims to increase palm oil production in order to serve the biodiesel industry, which has gained greater importance recently due to a sharp rise in global oil prices, which necessitates the finding of alternative energy sources. With a continuous rise in demand for biodiesel, it was estimated that domestic consumption of biodiesel will grow to 31.3 billion liters per year over the next six years. To accommodate the increased need for oil palm, by the year 2012 the area under cultivation will need to increase from the current to over nine million rai (Watcharas, 2008). The oil palm is especially valuable because of the large amount of oil produced in the oil palm fruit, a unique biological characteristic of this palm species (Hartley, 1977). Currently, all commercial oil palms are F1 hybrids (tenera) between pisifera (small or without kernels) and dura (large thick kernels), but the hybrids show a very high variation in oil yield with the best plants yielding 40% more than average. Improvement in oil palm production, especially for oil yield, has generally been achieved via conventional crosses between dura and pisifera cultivars. Tenera, an F1 hybrid resulting from this cross, contains desirable traits from both parents. As oil palm is normally propagated by seeds, a high variation in the field and available sustainable genetic trait production in traditional breeding is limited lead to the low palm oil yield. It is not only difficult to propagate true-to-type plants by seeds (because of the heterozygous nature of this species), but also vegetative propagation is impossible for oil palm. Therefore, it is necessary to develop vegetative propagation to produce large number of plants from elite palm. Nowadays, plant micropropagation is applied to plant

breeding in order to overcome some limitations, clonal propagation oil palm through tissue culture. (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. 2007). A reliable and efficient procedure for *in vitro* propagation of elite clones could significantly increase oil palm yields. Earlier studies from our laboratory were based on regeneration from leaf explants using dicamba (Te-chato *et al*., 2003; Promchan and Te-chato, 2007). However, a shortage of good hybrid oil palm species is the major problem of oil palm cultivation in Thailand. Therefore, it is necessary to verify the hybrid genetics at an early stage before commercial plantation to assure the uniformity and stability of the field performance and yield.

At present, molecular markers are used in many agricultural areas, especially plant breeding. Such markers are useful in analytic tests such as isozyme analysis that can be used for determination of hybridity. However, this method has limitations due to many factors affecting protein expression including development of plant tissue and the environment. A low number of markers restricted polymorphism may also affect the utility of these markers (Walter *et al*., 1989). RAPD (Randomly amplified polymorphic DNA) is a valuable tool for identifying genetic variation because it is economic, quick and simple (Williams *et al*., 1990). It permits identification of DNA polymorphisms and can be used to amplify particular fragments of genomic DNA (Bielawski *et al*., 1996). DNA fragment profiles have been employed to analyze the genetic relationships of plant species (Ayana *et al*., 2002). RAPD analysis is based on the presence or absence of polymorphisms in individuals or groups of individuals (Tingey and Tufo, 1993). Simple sequence repeat (SSR) or microsatellite is preferred for high throughput mapping, genetic analyses and marker assisted plant improvement programmes (McCouch *et al*., 2002). SSR had the highest information content (ability to distinguish genotypes) when evaluating soybean germplasm (Powell *et al*., 1996). The highly polymorphic nature of SSR is of particular interest in oil palm analysis, as oil palm breeding programmed often involve narrowly adapted gene pools. SSR markers, which

are PCR-based, have also been described as having the advantage of reliability, reproducibility, discriminative ability and cost-effectiveness when compared to other marker systems (Smith *et al*., 1997). Their high information content and the ease with which PCR assays can be automated for identifying SSR polymorphisms make SSR ideal genetic markers. Furthermore, since primer sequences are easy to share, this marker system is easily transferable from one laboratory to another. In addition, SSR markers have also been shown to have transferability across different species and plants, a fact that increases their value in plant genetic studies (Peakall *et al*., 1998). As co-dominant and locus specific markers, SSR have been widely used as tools in genotype identification and population genetic studies in plants.

Our attention was focused on the potential to obtain the use of half-embryo technique of six combinations of hybrid oil palm and verified their hybridity by RAPD and SSR analysis. Moreover, the left of half seed will be brought to induction of callus for micropropagation and completed plantlets were confirmed the uniformity before cultivation in the field.

Objectives

1. To screen and detect six combinations of hybrid with half-embryo technique by RAPD and SSR analysis.
2. To induce callus formation and germinated hybrid oil palm.
3. To confirm true-to type of completed plantlets derived from MZEs cultured.

CHAPTER 2

LITERATURE REVIEW

CHAPTER 2

LITERATURE REVIEW

2.1 Importance

Industrial exploitation of oil palm started at the beginning of the 20th century, initially in South-east Asia, later along the Gulf of Guinea in Africa and then in tropical America. During the latter half of the 20th century, its cultivation area extended in the Americas, mainly in the Amazon basin and along the Pacific coast of Colombia and Costa Rica. Intensive planting also occurred in South-east Asia and to a lesser extent in West Africa. Palm oil is the second largest source of edible oil in the world after soybean (Schwitzer, 1980). It is used as liquid cooking oil and in the manufacture of shortenings, margarines and spreads (Corley and Tinker, 2003). Palm oil contributes approx. 20% of world oil and fat production. It is projected that the demand for oil will soon exceed supply (Oil World Annual, 2001). By the year 2020, it is expected that nearly 26% of the world's oil and fat demand will have to be met by palm oil and it will capture approx. 50% of the world's oil and fat trade (Rajanaidu and Jalani, 1995). World palm fruit production has increased tenfolds (143,389,800 tones) since 1948 (FAOSTAT, 2004). Malaysia and Indonesia together account for more than 80% of world production, with an average increase of 7% between 1995 and 2000, while production remained static in South and Central America, particularly in Colombia. The consumption of oleaginous products continues to rise due to demographic pressure, and increased domestic use of oil palm in producing countries.

2.2 The classification of oil palm

The family of palms, the Arecaceae (formerly known as the Palmae), has always formed a distinct group of plants among the monocotyledons. The Arecaceae are placed in the order Arecales (Cronquist, 1981), although Bentham and Hooker's

Genera plantarum placed the palms with the Flagellariaceae and Juncaceae under the series Calycinae, while Engler and Prantl's system placed them separately under the order Principes. The oil palm, *Elaeis guineensis* Jacq., is grouped with *Cocos* (the coconut) and other genera in the subfamily Cocosoideae. The genus *Elaeis* was based on palms introduced into Martinique, the oil palm receiving its botanical name from Jacquin (1763) (Corley and Tinker, 2003). *Elaeis* is derived from the Greek word *elaion*, meaning oil, while the specific name *guineensis* shows that Jacquin attributed its origin to the Guinea coast. There are currently three accepted species of *Elaeis*. The first two species are *E. guineensis* and *E. oleifera*, the African and American oil palms. The third species was *Barcella odora* and now rename by Wessels-Boer (1965) as *Elaeis odora* (Corley and Tinker, 2003); it is not cultivated, and little is known about it. Henderson (1986) illustrated the bisexual inflorescences, and considered that the species was quite distinct from *Elaeis*. However, molecular markers indicated that inclusion of *E. odora* within the genus *Elaeis* is justified (Barcelos *et al*., 1999): the genetic distance between *E. odora* and the other two species of *Elaeis* was similar to the distance between the latter two species, and less than the distance from *Cocos nucifera*, another member of the Cocosoideae subfamily. From time to time other specific names have been attached to supposed species of *Elaeis*, but none has shown any signs of permanency other than *E. melanococca*, now named *E. oleifera*, and *E. madagascariensis*. *E. madagascariensis* Becc. was described by Beccari (1914) (Corley and Tinker, 2003). This material was distinguished from *E. guineensis* on some flower and fruit characteristics, but in view of the wide variation in many minor characters in the oil palm it is doubtful whether these differences justify the namine of a separate species. Based on observations in the field in Madagascar, Rajanaidu (1987) considered that specific status was not justified (Corley and Tinker, 2003).

2.3 Morphological characteristic of oil palm

E. guineensis has 16 pairs of chromosomes (diploid number, 2n = 2X = 32) and is a large, pinnate-leaved palm having a solitary columnar stem with short

internodes. There are short spines on the leaf petiole and within the fruit bunch. The separate upper and lower ranks of leaflets on the rachis give the palm a characteristic untidy appearance. Adventitious root system, forming a dense mat in the upper 35 cm of the soil, with only a few roots penertraing deeper than 1 m Stem cylindrical, up to 75 cm diameter, covered with petiole bases in young palms, smooth in older trees (10-12 years old). Juenile leaves are of lanceolate type, entire to gradually becoming pinnate; mature leaves are spirally arranged, paripinnate, up to 7.5 m long; petiole 1-2 m long, spinescent, clasping the stem at base; leaflets linear, 35-65 cm × 2-4 cm up to 376 per leaf (Figure 1). Inflorescences unisexual, axillary, pedunculate, unit anthesis enclosed in 2 uniforms or ovate spathes 10-30 cm long, with flowers 3-merous; male ones with numerous cylindrical spikes forming an ovoid body of 15-25 cm long and bearing flowers with 6 stamens, connate at base, with linear anthers; female ones subglobose, 15-35 cm diameter, with numerous lanceolate, spiny bracts, each subtending a cylindrical spikelet with 10-20 spirally arranged female flowers, each with two rudimentary male flowers; stigma sessile, 3-lobed. Fruits ovoid-oblong drupes, 2-5 cm long, tightly packed in large ovoid bunches with 1,000 to 3,000 fruits (Figure 2); drupes with a thin exocarp, an oleifeous mesocarp and a lignified endocarp containing the kernel with embryo and solid endosperm. In common with *E. guineensis* was considered from the occurrence of three natural fruit types under monogenic control. Pisifersa is homozygous (sh^{-} sh^{-}) for the absence of an endocarp. Dura is homozygous (sh^{+} sh^{+}) for the presence of a relatively thick endocarp (shell 2-8 mm) and less mesocarp. Tenera is heterozygous (sh^{+} sh^{-}) with a relatively thin endocarp (0.5-4 mm) and more mesocarp (Moretzsohn *et al*., 2000). Tenera is a hybrid from obtained by crossing Dura (female) and Pisifera (male).

This enables the production of uniform plantlets from any selected elite palm. The variation due to genetic differences could be avoided if oil palm was propagated clonally. Moreover, it is estimated that high yields and improved oil quality would be obtained by cloning palm processing the desired characters. Clonal propagation also has the advantage that secondary characteristics such as reduced

plant height and superior quality, found in selected individual palm, could be uniformly expressed in all individuals and ramets of the respective clones.

Figure 1 The oil palm plant at Klong Hoi Khong, Agricultural Research Station, Klong Hoi Khong, Faculty of Natural Resource, Hat Yai, Songkhla, Thailand

Figure 2 Mature oil palm fruits bunch, Tenera cultivar at Klong Hoi Khong, Agricultural Research Station, Hat Yai, Songkhla, Thailand

2.4 Oil palm tissue culture

Oil palm cannot be vegetatively propagated by conventional means, despite attempts to reverse floral bud differentiation and manipulation of vivipary (Davis, 1980). Excision of the apex results in death of the mother tree and *in vitro* culture of apices was attempted with young palms (Staritsky, 1970), but the results were unsatisfactory. Although seed of *E. guineensis* can be propagated but also seed propagation is poor germination due to dormancy of zygotic embryo. Dormancy is a state in which there is a block to germination due to some inherent inadequacy of the mature embryo, which may be manifested only when the embryo suffers some external constraints such as that imposed by the seed coat or other enclosing tissues (Ndon, 1985) and by the kernel, so-called kernel factors (Hussey, 1958; Te-chato, 1998a). Hussey (1958) showed that the embryo itself is not dormancy, but starts to elongate immediately after removed from the kernel which is the factors involved and controlled the germinating processes. Therefore, the critical requirement to break dormancy was to maintain the seed at temperature of 39-40°C for

up to 80 days. The rate of growth was low, compared with normal germination, but others have shown that excised embryos will grow well on a suitable culture medium (Rabechault, 1962; Corley and Tinker, 2003).

Attempts to propagate oil palm by tissue culture started in the 1960s, and by the mid-1970s success had been achieved (Jones, 1974). The early work was reviewed by Corley (1982) and Jones (1990). The first clonal palms were planted in the field in Malaysia in 1977 and the first replicated trials in 1978 (Corley, 1979). 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 (Corley, 1982). 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 status of the explant (Chehmalee and Te-chato, 2007), the composition and concentration of the basal salt (Te-chato *et al*., 2003), the organic components and plant growth regulators (Thawaro and Te-chato, 2007) in culture medium. Among these most importance factors, genotype and culture medium are discussed.

2.4.1 Genotype

Genotype is the most important factor influencing embryogenic response. Variability in the induction and frequency of the obtained embryos is observed among different species of genera and within the cultivars (Corley and Tinker, 2003). Considerable variations in embryogenic capacity were also observed between individuals of one cultivar or species. Genotype-dependent embryogenic capability was widely reported. Sanputawong and Te-chato (2008) reported that

different genotypes gave the different response on the percentage of cultures producing callus, embryogenic callus and number of embryogenic callus per explant. This result indicated that the highest percentage of callus formation was obtained from cross number 7 [derived from 1514 (D) × 521 (P)] and percentage of embryogenic callus formation (18) was obtained from cross number 14 [derived from 12 (D) × 38 (P)]. The highest number of embryogenic callus formation per explant was obtained from cross number 16 [derived from 1514 (D) × 38 (P)] after 3 months of culture, significant difference to others crosses. Genotype has also been found to affect both germination and callus formation of oil palm. Chehmalee and Te-chato (2007) also found that the highest percentage of germination was obtained in 366 (D) × 110 (P) cross combination after cultured on growth regulator-free MS medium when compared with 865 (D) × 110 (P) cross combination. Genotypic differences in plant regeneration have been observed in a wide range of species. Some genotypes exhibit high regenerative capacity, while others are either recalcitrant or irresponsible (Shen *et al*., 2008). Phillips (2004) reported specific genes are involved in each stage of plant regeneration (dedifferentiation, acquisition of competence, and induction). In some genotypes, genes involved in plant regeneration may be suppressed due to inappropriate culture medium and culture condition. Recently, Rival (2000) reviewed regeneration protocols for oil palm of different genetic origins. The frequency at which proliferating embryogenic cultures (PEMs) are obtained, enabling mass production at 40% and is currently the major stumbling block for large-scale ramet production for many selected ortets.

Various explants have been used to initiate oil palm *in vitro* cultures such as immature leaf (Te-chato *et al*., 1998a; Te-chato *et al*., 1998b; Te-chato *et al*., 2003), immature inflorescence (Wooi *et al*., 1981; Teixeira *et al*., 1994), the tips of tertiary roots (Kerslake, 1984; Mayes *et al*., 1995; Mayes *et al*., 1996) and zygotic embryos (Rabechault and Cas, 1972). Especially zygotic embryos, it can be produced mass scale propagation of oil palm seedlings in a short period of time. Therefore, embryo culture has great potential for improving the efficiency clonal propagation of oil palm (Zhang *et al*., 2003; Chehmalee and Te-chato, 2007).

2.4.2 Culture media composition

Plant tissues and organs 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;

2.4.2.1 Relatively large amounts of some inorganic elements (the so-called major plant nutrients): ions of nitrogen (N), potassium (K), calcium (Ca), phosphorus (P), magnesium (Mg) and sulphur (S).

2.4.2.2 Small quantities of other elements (minor plant nutrients or trace elements): iron (Fe), nickel (Ni), chlorine (Cl), manganese (Mn), zinc (Zn), boron (B), copper (Cu), and molybdenum (Mo).

The elements listed above are - together with carbon (C), oxygen (O) and hydrogen (H) - the 17 essential elements. Certain others, such as cobalt (Co), aluminium (Al), sodium (Na) and iodine (I), are essential or beneficial for some species but their widespread essentiality has still to be established. Culture medium was developed for optimal growth and the development of plant involved a large number of dose-response curves for the various essential minerals. Medium salt composition has also been shown to affect the formation and further development of somatic embryos. Culture media also play significant role on plant development. In oil palm tissue culture, mostly authors reported the use of basal MS Murashige and Skoog (1962) (MS) or modifications of this medium, half strength MS (½ MS), Y3 (Eeuwens, 1978) and Blade (BL) medium as the proliferation and germination medium. Generally, germination of seedling needs only the importance basal medium. MZEs capable of synthesize plant growth regulator and has normal germination under field condition. In case of culturing MZEs, only basic nutrients for germination are necessary due to removal of food storage tissue. Patcharapisutsin (1990) reported that 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. In the same year, result in comparison of oil palm embryos cultured on various culture media showed that Y3 medium supplemented with

0.05% activated charcoal gave the best seedling formation (Chourykaew, 1990). Singlaw (1989) found that the culture of MZEs in basal MS liquid medium for 10 days gave germination at 98%. Srisawat and Kanchanapoom (2005) reported that MZEs cultured on hormone-free MS medium gave the higher percentage of germination than hormone-free Y3 medium. Chehmalee and Te-chato (2007) also found that MZEs of all crosses cultured on growth regulator-free MS medium gave the highest percentage of germination at 16.67 when compared with other culture media. Addition of arginine and glutamine to the medium promoted germination due to the increase in amount of soluble protein and of 7S globulin per embryoid, but levels remained much lower than MZEs (Morcillo *et al*., 1999). Thus MS medium widely is the standard media of cultured tissues and has the sufficient nutrient requirement of oil palm germination. However, not only culture medium plays role on germination, but also genotypes and plant growth regulators influence on available for germination.

2.4.3 Plant growth regulators

Some chemicals occurring naturally within plant tissues (so called endogenously), have a regulatory, rather than a nutritional role in growth and development. These compounds, which are generally active at very low concentrations, are known as plant hormones (or plant growth substances). Synthetic chemicals with similar physiological activities to plant growth substances, or compounds having an ability to modify plant growth are usually termed plant growth regulators. Some of the natural growth substances are prepared synthetically or through fermentation processes. When these chemicals have been added to plant tissue culture media, they are termed plant growth regulators, to indicate the fact that they have been applied from outside the tissues (so called exogenously). The most plant proliferation and regeneration have been used the auxins, 2,4-dichlorophenoxyacetic acid (2,4-D) (Ward and Jordan, 2001; Satyavathi *et al*., 2004), 1-naphthaleneacetic acid (NAA) (Te-chato, 1998b; Chamalee and Te-chato, 2007) and 3,6-dichloro-o-anisic acid (dicamba) (Castillo *et al*., 1998; Can *et al*., 2004). The use of PGRs in oil palm is generally depending on the type of explants

and the genetic origin of the material. 2,4-D in the initiation media was essential for callus induction from mature zygotic embryos (MZEs) culture of oil palm (Teixeira *et al*, 1993). Teixeira *et al.* (1994) also reported that immature inflorescences were cultured on 2,4-D containing medium gave the highest callus induction at 50%, in comparison with other concentrations. Teixeira *et al.* (1995) also found that immature zygotic embryos (IMZEs) were cultured on 2,4-D containing medium gave the friable callus induction enough for establishing cell suspension cultures. Although numerous workers have successfully using 2,4-D for callus induction, very high concentration required. The high concentration of 2,4-D was reported to increase chromosome instability, leading to somaclonal variation (Karp, 1994). Therefore, other type of auxins such as dicamba has been recommended (Pedrosa and Vasil, 1996; Te-chato, 1998a). Due to 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). Oil palm cultures do not require exogenous cytokinins for growth, and growth is not stimulated by adding cytokinins (Nwanko and Krikorian, 1983). However, the addition of 1-5 mg/l BA increased germination rates of embryos in embryogenic suspension up to 70% (Aberlance-Bertossi *et al*., 1999). Among cytokinin KN, BA and 2-iP at very low concentration were added to proliferation medium (Rabechault and Martin, 1983). Generally, cytokinins promote cell division and cell expansion in plant cell culture. In clonal propagation of tropical fruit trees, growth is stimulated by adding a cytokinin to a proliferation medium. Many authors reported the suitable cytokinin types and their concentration for fruit crop species (Te-chato and Lim, 1999; 2000). Cytokinins are classified into two major groups: synthetic phenylurea derivatives, such as 1-phenyl-3-(1,2,3-thiadiazol-5-yl) urea known as thidiazuron (TDZ) and *N*-(2-chloro-4-pyridyl)-*N'*-phenylurea (CPPU) and adenine derivatives, which may occur naturally, such as kinetin (KN) and 6-benzyladenine (BA). Synthetic phenylurea derivatives, especially TDZ, have activities higher that adenine derivative (Mok *et al.,* 1987; 2000). BA is used to micropropagate a wide array of plant species, including woody plants, because of its great ability to stimulate shoot proliferation and regeneration (Montoro *et al*., 1992;

Verdeil *et al*., 1994). Te-chato *et al*. (2008) reported that the combination of KN (0.1 mg/l) with dicamba (0.1 mg/l) gave small aggregates composed of round, dense in cytoplasm led to establishment of fine suspensions in short period of time. The two PGRs were proved to play an important role in both cell viability and formation of chloroplasts in embryogenic cell suspension culture from young leaf-cultured of oil palm.

2.5 Review of oil palm *in vitro* culture works

The application of tissue culture to oil palm production has been ongoing for three decades. The first pioneer excised zygotic embryo of oil palm for culture in culture medium in the presence of auxin by Rabechault *et al*. (1976). This result has revealed that the embryos can both germinate and produce callus (Te-chato, 1998a). Cui *et al*. (1988) have also reported callus induction from culturing MZEs of oil palm in culture medium. Jones (1990) found that the endogenous cytokinins in embryoids and MZEs differed, with generally much higher levels in the latter. Morcillo *et al*. (1998) observed that embryoids accumulated the same storage protein (7S globulin) as MZEs, but at less than 2%. Teixeira *et al*. (1993) determined that 2,4-D in the initiation media was essential for callus induction from culturing MZEs. An optimal level was 500 μM. Teixeira *et al*. (1995) also found that IMZEs were cultured on 475 μM 2,4-D containing medium gave the friable callus induction. Chehmalee and Te-chato (2007) found that all MZEs crosses cultured on growth regulator-free MS medium gave the highest percentage of germination at 16.67 when compared with other culture media. Using leaf explants, all palm samples gave callus, with the proportion of mature palm explants giving callus ranging from 7 to 60% (Wong *et al*., 1999b; Rival, 2000). Ortets of Deli × La origin gave higher callus frequency than Deli × Yangambi or Deli × NIFOR (Rival, 2000). Te-chato *et al*. (2008) reported that the combination of kinetin (0.1 mg/l) with dicamba (0.1 mg/l) were proved to play an important role in both cell viability and formation of chloroplasts in embryogenic cell suspension culture from young leaf-cultured of oil palm. For immature inflorescences explant, Teixeira *et al*. (1994) reported that immature

inflorescences were cultured on 500 μM 2,4-D containing medium gave the highest callus induction at 50%, in comparison with other concentrations. Some preliminary work on culture of oil palm microspores has been published (Odewale, 1989; Latif, 1991; Tirtoboma, 1998), but as yet there are no reports of homozygous diploid plants being produced. Jones (1989) discussed the problems of testing the homozygous *duras* and *teneras* that might be developed from anther cultures. Such palms could not be judged on phenotypic performance, but progeny testing even relatively small numbers would involve several hundred hectares of trials. In case of root explant, Wooi (1995) found no difference in callus frequency from root explants between palms aged 9–10, 14–16 and 22–23 years, but 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.

2.6 Molecular markers technologies in oil palm

The first work on molecular markers was with proteins some enzymes exist in more than one form (isoenzymes or isozymes), and these forms can be separated and their inheritance studied. Some work has been done with oil palm (Ghesquiere, 1984, 1985; Ataga and Fatokun, 1989b; Baudouin, 1992; Choong *et al*., 1996), but protein markers are difficult to work with. The enzymes present differ between tissues, and even within the same tissue may vary with physiological status of the plant, so it can be difficult to obtain comparable samples from different plants; also, there are relatively few enzymes for which the isozymes are easily separated and identified. The most reliable markers are those based on DNA; these depend on the distinctive structure of the genetic material, and have largely replaced protein markers in genetic studies. A large proportion of the DNA of most plants, including oil palm, consists of repetitive sequences separating the genes. Many of these repetitive sequences have not known function, but their frequency makes them useful in marker studies. DNA markers have new become a popular means of identification of plant species.

DNA-based markers are less affected by ages, physiological condition of samples and environment factors. They are not tissue-specific and thus can be detected at many phase of organism development. Only a small amount of samples is sufficient for analysis and the physical forms of the sample dose not restrict detection.

2.6.1 RAPD marker

RAPD procedure is technically the simplest version. It employs single primers with 10 nucleotides and a GC content of at least 50%. PCR products are separated on agarose gels and detected by staining with ethidium bromide (Williams *et al.*, 1990). Given that RAPD primer sequences are arbitrarily chosen, the genome is expected to be sampled randomly. Most RAPD fragments are inherited as dominant markers, i.e., they are either present or absent. A fragment is seen in the homozygous (AA) as well as in the heterozygous (Aa) situation, and only the absence of the fragment clearly reveals the underlying genotype (aa). Williams *et al*. (1990) and Fritsch and Rieseberg (1992) found that at least 95% of RAPD fragments were dominant markers, whereas the remaining behaved co-dominant, as two alleles with a different size. The greatest advantage of the RAPD approach is its technical simplicity, paired with the independence of any prior DNA sequence information, less costly and less labor intensive than other DNA marker methodologies. RAPD is a valuable tool for identifying genetic variation because it is economic, quick and simple (Williams *et al*., 1990). It permits identification of DNA polymorphisms and can be used to amplify particular fragments of genomic DNA (Bielawski *et al*., 1996). DNA fragment profiles have been employed to analyze the genetic relationships of plant species (Ayana *et al*., 2002). RAPD analysis is based on the presence or absence of polymorphisms in individuals or groups of individuals (Tingey and Tufo, 1993). RAPD markers have been employed in the analysis of genetic variation among African germplasm accessions of oil palm (Shah *et al*., 1994), in attempts to detect somaclonal variants among regenerant populations of oil palm (Rival *et al*., 1998) and in the identification of markers linked to the shell thickness locus of *E. guineensis* (Moretzsohn *et al*., 2000). Moretzhn *et al.* (2002) found

that two RAPD markers (R11–1282 and T19–1046 primers) were identified to be linked on both sides of the sh^{+} locus on linkage. In additionally, primer UBC731 could be identified the cultivars and clone of oil palm by Kanop *et al.* (2005).

2.6.2 SSR marker

SSR is tandem arrays of simple nucleotide motifs that are ubiquitous components of eukaryotic genomes (Delseny *et al*., 1983; Tautz and Renz, 1984; Tautz, 1989). Inherited in a Mendelian fashion (Weissenbach *et al*., 1992; Saghai Maroof *et al*., 1994), their hypervariable length polymorphism is revealed by the polymerase chain reaction (PCR) using flanking primers that generate co-dominant markers. According to Smith *et al*. (1997), SSR technology is reliable, reproducible, discriminating and cost effective. Development of oil palm microsatellite markers has been used for measuring genetic diversity, variety identification, pedigree analysis and genome mapping and for quality trait loci (QTL) detection for MAS (Jones, 1989; Brown, 1993; Jack and Mayes, 1993; Mayes *et al*., 1996a,b). Billotte *et al*. (1999) described an efficient technique for building microsatellite enriched libraries. This technique allowed the construction of several oil palm $(GA)_n$, $(GT)_n$ or $(CCG)_n$ enriched-libraries from total genomic as well as chloroplast DNA (cpDNA). About 200 functional SSR primer pairs have already been developed from microsatellite clones and have been sequenced. Billotte *et al*. (2001) characterized 21 SSR loci together with primer sequences; estimates of allele size range were made, as well as expected heterozygosity in *E. guineensis* and in the closely related species *E. oleifera,* where an optimal utility of the SSR markers was observed. Multivariate data analyses indicated that SSR markers can reveal the genetic diversity within the genus *Elaeis* in accordance with known geographical origins and measured genetic relationships based on previous molecular studies. High levels of allelic variability indicate that *E. guineensis* SSRs are putative markers for all palm taxa. In addition, phenetic information based on SSR flanking region sequences makes *E. guineensis* SSR markers a potentially useful molecular resource for study of palm taxa phylogeny. SSRs will be powerful for genetic studies of the *Elaeis* genus for variety

identification and intra specific or interspecific genetic mapping. PCR amplification tests, from a subset of 16 other palm species, and allele sequence data show that *E. guineensis*. SSRs are putative markers for all palm taxa. In addition, phenetic information based on SSR flanking region sequences makes *E. guineensis* SSR markers a potentially useful molecular resource for study of palm taxa phylogeny.

CHAPTER 3

Propagation of Hybrid Oil Palms by Culturing of Zygotic Embryos

Experiment I

Effect of genotypes and concentrations of various auxins on callus formation

CHAPTER 3

EXPERIMENT I

Introduction

In vitro culture of mature embryos is the method of choice for producing abundant callus and sufficient shoot formation in vegetative propagation of many plants including palms. Callus is an unorganized mass of plant cell for inducing *in vitro* plant regeneration and successful application of direct gene transfer techniques. The stimulation of cell division and callus formation is frequently a result of a complex interaction between endogenous and exogenous growth substances (Salehi and Khosh-khui, 2005). In addition, the ability of callus induction is genotype depend on the following factors: (Halamkova *et al*., 2004 and Shen *et al*., 2008), genetic background of donor plant (Can *et al*., 2004 and Kilinc, 2004) and plant growth regulators (PGRs) (Bregitzer, 1992; Dahleen, 1995; Castillo *et al*., 1998; Jiang *et al*., 1998). In oil palm, both of genotype and PGRs are being the most important that affect the efficiency of *in vitro* culture responses. Genotypic response in callus formation has been observed in mature zygotic embryos (MZEs) of oil palm. Sanputawong and Te-chato (2008) reported that callus formation capacity of 16 crosses of oil palm were distinctly different. For example, cross number 7 gave the highest percentage of callus formation at 33.33, significant difference to others crosses. Chehmalee and Te-chato (2007) found that hybrid seed of 366 (D) × 110 (P) cross combination gave the higher percentage of callus formation (54.44) than cross 865 (D) × 110 (P) (35.18%). PGRs also influence upon the capacity for callus formation, which may be related to the totipotency of cells at their developmental stages (Agarwal and Ranu 2000; Bacchetta *et al*., 2003). Additionally, the type, concentration, and combination of PGRs in the culture media can greatly affect callus formation (Mithila *et al*., 2003 and Thao *et al*., 2003). The auxins, 2,4-dichlorophenoxyacetic acid (2,4-D) (Ward and Jordan, 2001; Satyavathi *et al*.,

2004), 1-naphthaleneacetic acid (NAA) (Te-chato, 1998b; Chamalee and Te-chato, 2007) and 3,6-dichloro-o-anisic acid (dicamba) (Castillo *et al*., 1998; Can *et al*., 2004) are the most commonly used for callus induction. In case of oil palm, Teixeira *et al.* (1993) pointed that 2,4-D in the initiation media was essential for callus induction from MZEs and the optimal level was 500 μM. Teixeira *et al.* (1994) also reported that immature inflorescences cultured on 500 μM 2,4-D containing medium gave the highest callus induction at 50%. The immature zygotic embryos (IMZEs) cultured on 475 μM 2,4-D containing medium gave the friable callus induction suit for establishment of cell suspension cultures Teixeira *et al.* (1995). Te-chato *et al.* (1998a) reported that among three levels of 2,4-D tested, 2.5 mg/l 2,4-D produced the best result in callus induction at 40%. Lower concentration of 1 mg/l 2,4-D yielded callus at lower frequency at 20%. Concentration of 2,4-D at higher than 5 mg/l could not induce callus formation. Although numerous workers have successfully used 2,4-D for callus induction, a high concentration was reported to increase chromosome instability, leading to somaclonal variation (Karp, 1994). The other type of auxins such as dicamba has also been reported on callus induction in oil palm (Pedrosa and Vasil, 1996; Te-chato, 1998a) and it was superior to other auxins while NAA gave the poorest result in callus formation (Te-chato, 1998b). In comparison with 2,4-D, dicamba was the best auxin source for culturing young leaves of oil palm. Chehmalee and Te-chato (2007) also reported that dicamba at concentration of 2.5 mg/l containing MS medium gave the highest percentage of callus formation (54.44). 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*., 2003).

The determination of histology of origin of callus through histological aspect is considerably inadequate. Few histological studies have dealt with palms (Tisserat and DeMason, 1980; Pannetier and Buffard-Morel, 1982; DeMason, 1988). Hence, the purposes of this chapter are to evaluate the effect of genotype, various kinds and concentrations of auxins on callus formation. 2) To described the origin of callus formation from MZE culture of hybrid oil palms.

Materials and methods

Plant material

Mature fruits from six oil palm hybrids from the following crosses; 366 (D) × 172 (P), 366 (D) × 72 (P), 366 (D) × 206 (P), 865 (D) × 206 (P), 865 (D) × 110 (P) and 366 (D) × 777 (P) were used in this experiment. All hybrids seed were kindly provided by Assoc. Prof. Dr. Theera Eksomtramage (Agricultural Research Station, Khlong Hoi Khong, Hat Yai, Songkhla, Thailand). All seeds were extracted from the fruit, cracked by hammer and trimmed by pruning scissors to remove the excess kernel. MZEs surrounded by kernel in cube of 3×3×3 cm^3 were sterilized in 70% alcohol for two min followed by 20% (w/v) sodium hypochlorite together with two to three drops of Tween-20 for further 20 min. The cubes were then thoroughly washed in sterile water for three times. The embryos were excised from the cubes and cultured on culture media.

Effect of genotypes and concentrations of various auxins on callus formation

Sterilized MZEs were placed into culture tubes (25×150 mm) containing 10–15 ml of modified MS (Murashige and Skoog, 1962) medium supplemented with either 40 mg/l NAA or 2,4-D or dicamba at concentration of 2.5 and 5.0 mg/l. All plant growth regulator (PGR) containing culture media were adjusted pH to 5.7 with 0.1 N KOH before adding 0.7% agar and autoclaving at 1.05 kg/cm^2, 121^{o}C for 15 min. The cultures were placed under light conditions of 40 $\mu mol/m^2/s$ for 16 h photoperiod at 25±2^{o}C and subcultured every 4 weeks on the same medium component for 3 months.

Histological Observation

For histological study, all types of callus formation were collected, fixed in FAA II solution (formalin: glacial acetic acid: 70% ethanol 5:5:90 v/v), dehydrated using an ethanol-tertiary butanol series for 24 h and embedded in Paraffin (Paraplast).

Embedded tissues were sectioned at 6 μm and mounted on glass microscope slides. Paraffin was removed in a xylene-ethanol series; tissues were stained with saffranin and fast green. All sections were mounted with Permount and viewed under bright field illumination of compound microscope (Olympus). Histological analysis was carried out on representative samples of the callus induced from different kinds and concentrations of auxins.

Data analyses

For experimental design and statistical analysis, CRD with 4 replicates (each replicate consist of 10 embryos) was performed. The percentage of cultures that produced callus, types of callus and number of the embryos per tube were recorded every month after 1 to 3 months. The number of embryos was counted under stereomicroscope (Nikon, SMZU) and data were analyzed using analysis of variance (ANOVA).

Results

Effect of genotypes and concentrations of various auxins on callus formation

MZEs of all genotypes swell at 10-14 days after cultured and started to from callus at 4-5 weeks of culture. After 6 weeks of subculture onto various kinds and concentrations of auxins, almost auxins promoted callus formation form MZEs of all crosses. Four types of calluses could be distinguished; friable, compact, nodular and root-like structure calluses (Figure 3). The friable calluses were yellow, translucent and succulent. The compact calluses were white and compact. The nodular calluses were yellow or pale yellow and compact (Figure 3A). The root-like structure calluses were elongate in shape, white color and soft texture (Figure 3B). Kinds and concentrations of auxins containing in culture media had significantly affect on type of calluses. Type of the callus obtained in NAA, 2,4-D and dicamba containing the medium were quite

different. Dicamba provided a yellow compact callus (so called nodular callus) whereas 2,4-D gave both a white friable callus and a white elongate soft callus. For NAA, it could not induce callus formation. The highest frequency of callus formation was obtained on MS medium supplemented with 2.50 mg/l dicamba, significant difference to other kinds and concentrations of auxins (Table 1).

In the present study, three different synthetic auxins (NAA, 2,4-D and dicamba) were compared. The result of our experiments showed that among genotypes tested, cross #58 derived from 366 (D) × 172 (P) gave the highest percentage of nodular callus at 48.50% and average number of nodule at 18.78±17.49 nodule/callus, significant difference to others genotypes when cultured on 2.50 mg/l dicamba containing medium (Table 1, Figure 4).

Histological observation

Histological study of the callus revealed that 2, 4-D containing medium induced nodular structure from epidermal cells of MZE (5A) while dicamba induced from both the epidermis and vascular tissue (5B) after 4 weeks of culture. These cells were small, cytoplasm dense and well stained nucleus. They proliferated intensely which caused a rupture of the epidermis and hence emergence of the nodular structure at the surface of zygotic embryo. In case of NAA and 2.50 mg/l 2, 4-D containing medium, nodular and elongate root-like structures (Figure 6A) arose from the epidermal layer of MZE (Figure 6B) and those structures developed into root primordia (Figure 6C) subsequent to root formation.

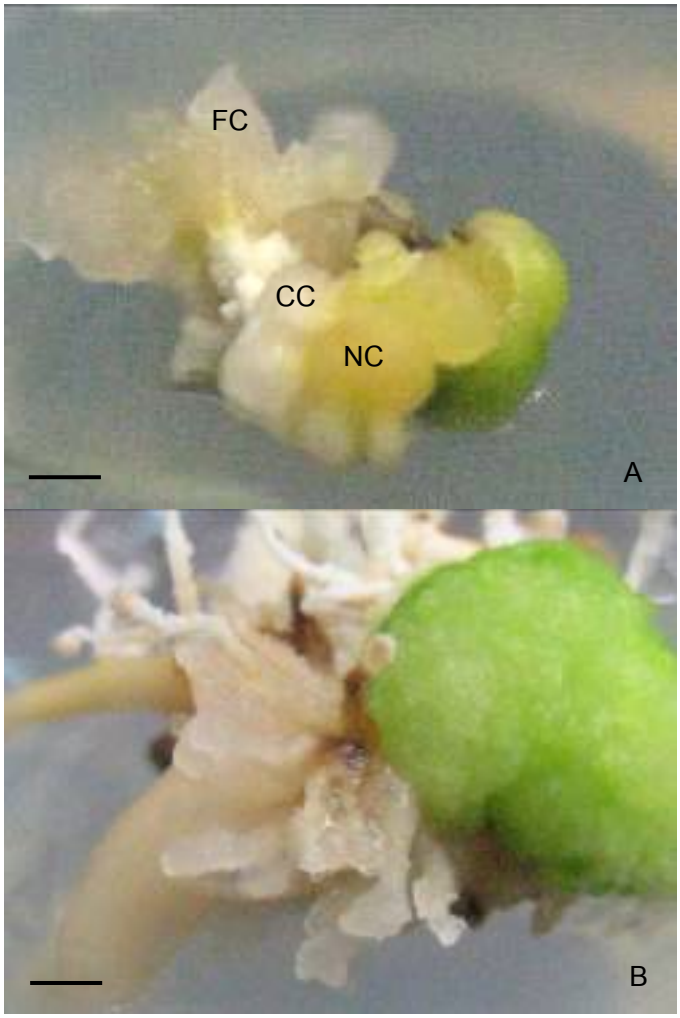


Figure 3 Cluster of friable callus (FC), compact callus (CC), nodular calluses (NC) (A) and root-like structure (B) from MZEs of hybrid oil palm cultured on solidified MS supplemented with various auxins for 3 months (bar = 2.50 mm).

Table 1 Effect of genotypes and concentrations of various auxins on percentage of type callus formation and type of callus from various crosses of hybrid of oil palm after culture on MS medium for 3 months.

Crosses(C)	Type of callus formation																				Mean$_{crosses}$
	Friable callus					Compact callus					Nodular callus					Root-like structure callus					
	Type of Auxins (A)																				
	NAA	2,4-D		Dicamba		NAA	2,4-D		Dicamba		NAA	2,4-D		Dicamba		NAA	2,4-D		Dicamba		
	Concentrations (mg/l) (B)																				
	40	2.5	5	2.5	5	40	2.5	5	2.5	5	40	2.5	5	2.5	5	40	2.5	5	2.5	5	
58	15e[1]	25de	20e	17.5e	22.5de	0g	2.5f	12.5e	15e	12.5e	2.5e	37.5d	70b	87.5a	45cd	27.5de	12.5e	52.5c	70b	22.5de	28.5A
77	15e	22.5de	20e	50c	10f	5f	7.5f	7.5f	12.5e	12.5e	2.5e	27.5de	35d	65b	10f	25de	10f	0g	0g	0g	16.88B
118	0g	23.57de	15e	33.3d3	28.3de3	0g	9.8f	5f	17.65e	16.67e	0g	39.22ce	20e	41.82d	41.18d	0g	7.84f	7.5f	15.69e	20e	17.13B
119	0g	29.09de	35.29d	34.6d5	29.13de	0g	8.18f	11.76e	11.65e	6.93f	0g	46.6ce	39.22d	50.19c	42.57d	27.5de	11.82e	13.73e	15.84e	12.62e	21.34AB
130	0g	12.5e	12.5e	15e	12.5e	0g	7.5f	10f	7.5ef	10f	0g	7.5f	40d	42.5d	12.5e	0g	10f	10f	15e	4.5f	11.48B
137	0g	17.5e	17.5e	37.5d	25de	0g	12.5e	12.5e	20e	10f	0g	37.5d	47.5cd	52.5c	45cd	0g	5f	7.5f	0g	0g	17.38B
Mean$_{concentrations}$	5E[2]	21.69CD	20.05CD	31.33C	21.24CD	0.83F	8E	9.88E	14.05D	11.43D	0.83F	32.64C	41.95B	56.59A	32.71C	13.33D	9.53E	15.21D	19.21D	9.94E	
Mean$_{auxins}$			19.86B					8.84C					32.94A					11.24C			*
C.V. (%)												44.14									

[1] a-g columnwise comparison (type and concentration of auxins combination within crosses),

[2] A-F rowwise comparion (crosses combination within type and concentration of auxins),

C.V. (%) is coefficient of variation, Different letter indicate significant differences at 5% level by DMRT

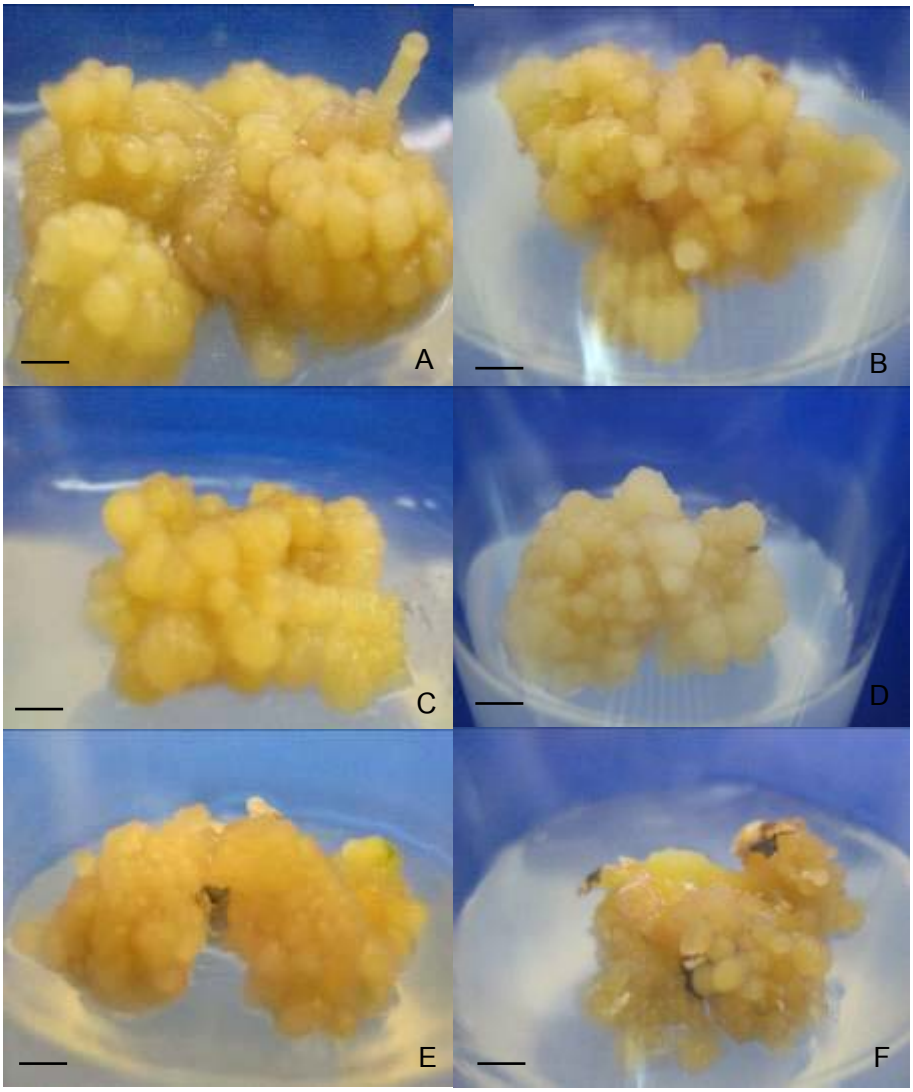


Figure 4 Nodular callus induction from cross #58 (A), cross #77 (B), cross #118 (C), cross #119 (D), cross #130 (E) and cross #137 (F) cultured on solidified with MS medium supplemented with 2.5 mg/l dicamba (bar = 2.50 mm).

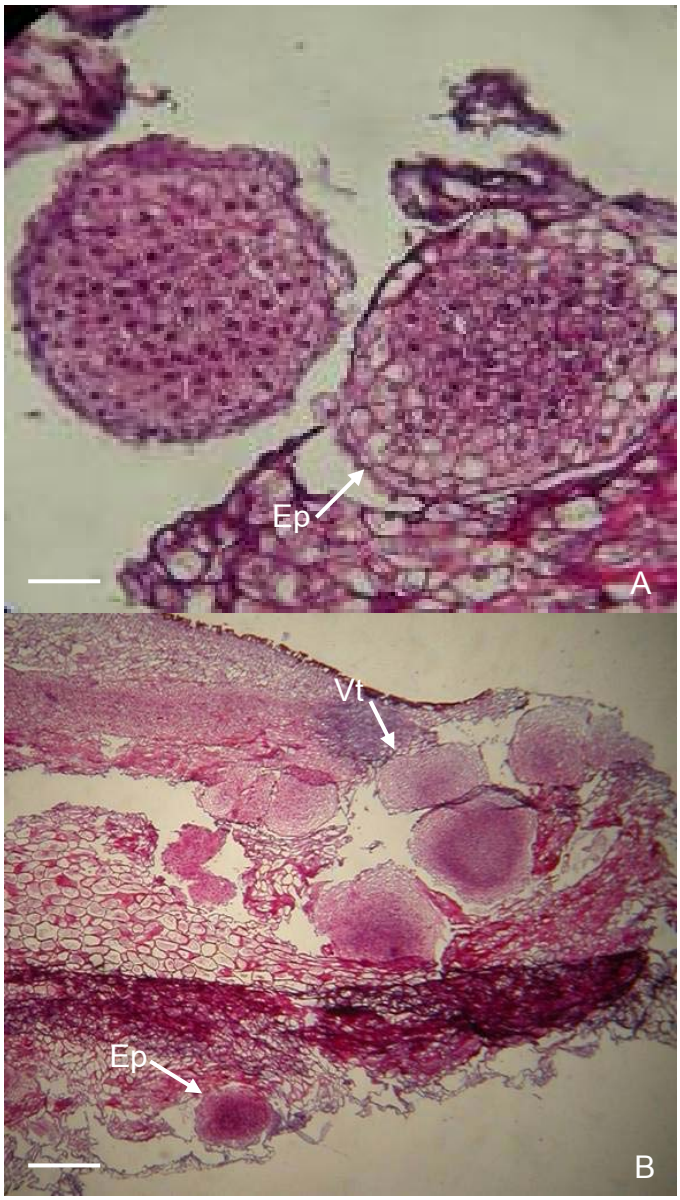


Figure 5 Histological study of callus in 2,4-D (A) or dicamba (B) containing medium. A: showed development of nodular structure from epidermis (Ep) alone. B: showed development of nodular structure from both epidermis (Ep) and vascular tissue (Vt) (bar = 50 μm).

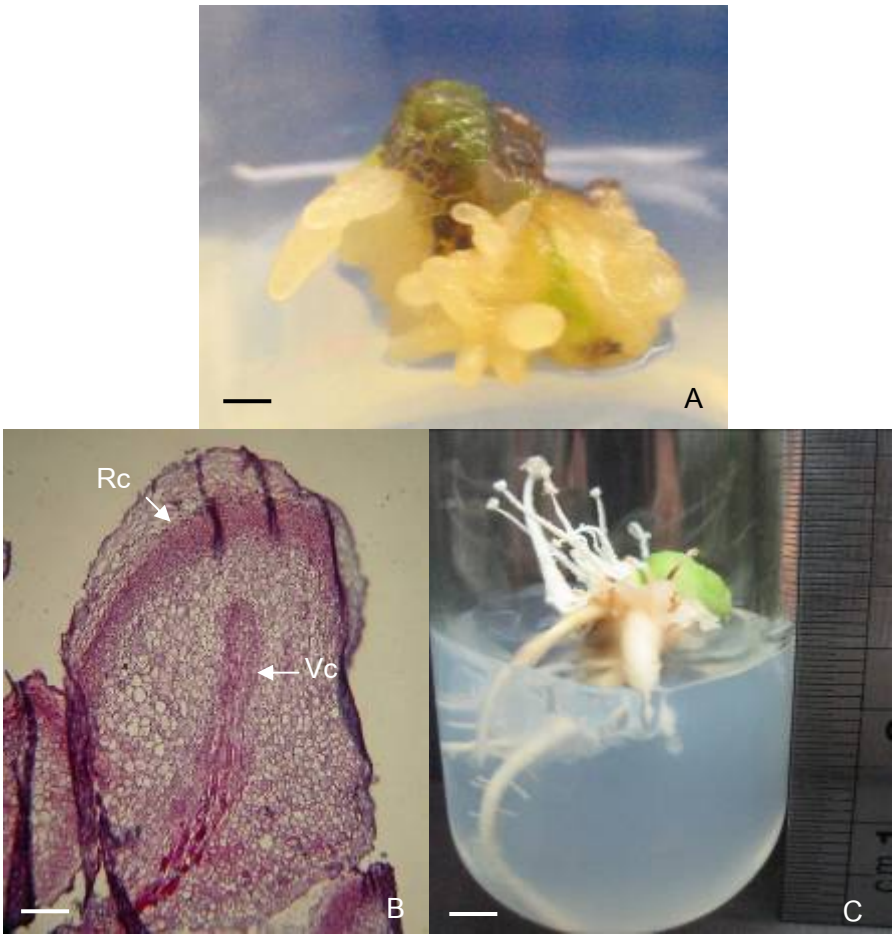


Figure 6 Morphological (A) and histological study (B) of callus in NAA or 2,4-D containing medium and development of root on the callus (C). Rc: root cap, Vc: vascular cambium (bar = 50 μm).

Discussion

Effect of genotypes and concentrations of various auxins on callus formation

Genotype is one of the most important factors influencing callus formation *in vitro*. Different responses can be observed among species, cultivars, and individuals (Brown, 1993). In this present study, efficient callus formation was achieved from MZEs of hybrids oil palm by manipulating different factors; genotype, kinds and concentration of auxin.

All genotypes of hybrid of oil palm provided high frequency of callus formation but different responses on type of callus were observed among auxin tested. Cross 58 gave the highest nodular callus formation and average number of nodular callus significant difference to others genotypes. Similar results were obtained by Chehmalee and Te-chato (2007) who has also found that the 366 (D) × 110 (P) cross combination gave the higher percentage of callus formation (54.44) than the 865 (D) × 110 (P) cross combination (35.18). Sanputawong and Te-chato (2008) also reported that callus formation capacity of 16 crosses of oil palm were markedly different. Cross number 7 [derived from 1514 (D) × 521 (P)] gave highest percentage of callus formation at 33.33, significantly different to others crosses. Niroula *et al.* (2005) and Filippov *et al.* (2006) reported that callus induction frequency are usually associated with the variations between genotypes in susceptibility to genetic programming and reprogramming of embryogenic competent cells by external factors. The difference in callus induction frequency may be due to seed vigor or size of MZE in seed like those reports of Fennel *et al.* (1996), Machii *et al.* (1998) and Ozgen *et al.* (1998).

In present work, the optimization of an external factor, such as the exogenous auxins, partially overcame the genotype influence and increased callus formation. This evident has been reported in numerous cultivars: *Feijoa sellowiana* (Guerra *et al.*, 2001), *Carthamus tinctorius* (Mandal *et al.*, 2001) and Rye (*Secale cereale* L.) (Ward and Jordan, 2001). In case of morphological characteristics of callus among 6 different genotypes induced from MZEs of hybrid oil palm, it showed that all

genotypes gave the different frequency of nodular callus which further developed into embryogenic callus. This ability might be depended on growing conditions of the donor plants. Similar results were observed by Rines and McCoy (1981) in oats, Duncan *et al.* (1985) in maize and Berthouly and Michaux-Ferrière (1996) in coffee. From this present study it is suggest that cross 58 have the hybrid vigor more than another crosses. The reason could be due to the good combining of gene between the two parents.

Kinds and concentrations of auxins supplemented in culture medium had a significant affect on type of calli. Characteristics of the callus obtained in NAA, 2,4-D and dicamba containing medium were quite different. The highest percentage of nodular callus was obtained from 2.50 mg/l dicamba. Similar result was also found in immature embryo culture of winter of wheat (Carman *et al.*, 1988) and spring wheat cultivar (Hunsinguer *et al.*, 1987). However, Morcillo *et al.* (2006) observed that 2,4-D was required for callus initiation in oil palm. The concentration of 2,4-D at 2.5 mg/l promoted the best callus formation in comparison within higher concentration of 2,4-D (5 mg/l). Similar result was observed by Kanchanapoom and Domyoas (1999). On the other hand, Teixeira *et al.* (1993) determined that 2,4-D in the initiation media was essential for callus induction from MZEs culture and the optimal concentration was 500 μM (105 mg/l). Teixeira *et al.* (1995) also found that immature zygotic embryos (IMZEs) were cultured on 475 μM (90 mg/l) 2,4-D containing medium gave the highest callus induction. Karp (1994) reported that the auxin at higher concentration is reported to increase chromosome instability, leading to somaclonal variation. In this present study, dicamba, a synthetic auxin, at low concentration was sufficient to initiate and promoted callus formation in oil palm and has been used as the only growth regulator in callus induction media (Te-chato, 1998a; Te-chato *et al.*, 2003; Sanputawong and Te-chato, 2008). Hutchinson *et al.* (1994) reported that dicamba treatment could result in an increase in endogenous auxin levels. This explanation may support the hypothesis why dicama was the most effective in callus induction in the present study. Moore (1989) suggested that dicamba is a highly stable auxin, showing strong resistance to enzymatic degradation and conjugation in plant cell. Dicamba is needed to induce

mitotic divisions of explant cells, especially in monocotyledon (Samai *et al.*, 2003; Filippov *et al.*, 2006).

Histological Observation

In this study, kinds and concentrations of auxins containing media had a significant effect on type of calli. However, type of the callus obtained in NAA, 2,4-D and dicamba containing medium were quite different. Origin of callus derived from zygotic embryo arose from single cell of epidermal layer alone in 2,4-D containing medium. A similar result was obtained in cultured zygotic embryo of oil palm, cv. tenera (Kanchancpoom and Domyoas, 1999). However, these results were contrary to Schwendiman *et al.* (1988) who reported that the origin of the oil palm somatic embryo was multi-cellular. Nwanko and Krikorian (1983) also studied the histology of callus derived from cultured zygotic embryos but they did not specify the origin of the embryos. In addition, Kanchanapoom and Tinnongjig (2001) reported that embryoids were derived from meristematic cells. The oil palm embryoids appeared to be similar to those described by DeMason (1988) (in palm *Washingtonia filifera*) and Tisserat and DeMason (1980) (in the date palm *Phoenix dactylifera*). It is probably true that the different species and sources of explant cause a different response in the origin of somatic embryos (Te-chato *et al.*, 2003). For dicamba containing medium it induced nodular structures from both the epidermal cells and procambium cells. Similar result was obtained in cultured primary callus derived from leaf fragment of oil palm. Te-chato *et al.* (2003) reported that meristemoid or nodule-like structures arose from both epidermal cell (epidermis layer) and parenchymatous cells (subepidermal layer) in dicamba containing medium. In case of NAA containing medium, root -like-structure callus arose from the epidermal layer of MZE and those structures developed into root primordia. This type of development was also evident in nodular structure obtained from 2.5 mg/l 2,4-D containing medium. By the results in this investigation, it suggested that dicamba was the most potent plant growth regulator for nodular callus induction both

qualitatively and quantitatively like those reported by Promchan and Te-chato (2007) and Te-chato and Hilae (2007). So, mass propagation of oil palm though tissue culture technique could be commercialized by this phytohormone.

In this present study it is clear evident that genotype plays role in type of callus. Cross #58 was classified as high capacity in callus induction, significantly different to other genotypes.

CHAPTER 3

Propagation of Hybrid Oil Palms by Culturing of Zygotic Embryos

Experiment II

Effect of genotypes on embryogenic callus formation, average number of embryogenic callus formation and type of somatic embryos

CHAPTER 3

EXPERIMENT II

Introduction

Plant embryogenesis begins with the zygote and passes through a stereotyped sequence of characteristic stages. Although considerable morphogenesis occurs after seed germination, the embryonic phase is crucial as it is here that meristems and the shoot-root body pattern are specified (Mordhorst *et al*., 1997). It is usually easy to distinguish between embryogenic and non-embryogenic callus based on morphology and colour. Embryogenic callus is composed of proembryogenic masses (PEMs). Besides, there are two mechanisms appear to be important for *in vitro* formation of embryogenic cells: asymmetric cell division and control of cell elongation (De Jong *et al.,* 1993; Emons, 1994). Asymmetric cell division is promoted by plant growth regulators (PGRs) that alter cell polarity by interfering with the pH gradient or the electrical field around cells (Smith and Krikorian, 1990). The ability to control cell expansion is associated with polysaccharides of the cell wall and corresponding hydrolytic enzymes (De Jong *et al*., 1993; Emons, 1994; Fry, 1995). Common regulatory mechanisms of embryogenesis were controlled by the differential expression of genes in embryogenic and nonembryogenic tissues. Studies have been conducted either to identify the genes expressed and gene products that accumulate specifically during different stages of embryogenesis or to analyze the expression of a variety of genes that probably have some role in the embryogenic pathway (Meinke, 1995). Embryogeic callus formation in oil palm has been documented from immature inflorescences (Teixeira *et al*., 1994), young leaf (Te-chato *et al*., 1998a; Te-chato *et al*. 1998b) and zygotic embryo derived callus (Nwanko and Krikorian, 1983; Rabechault and Cas, 1974). Those studies have contributed to the development of genetic engineering technology for the improvement of oil palm by protoplast or biolistic transformation. However, efficiency of embryogenenic callus formation and proliferation from oil palm is also not high enough

to achieve mass propagation. It has been known that embryogenic callus has been observed to be induced indirectly by different factors such as genotype, endogenous plant hormones, exogenous plant growth regulators (PGRs), and source of explant. Among those, genotype is the one of important factor influencing embryogenic callus. Genotypic response in embryogenic callus formation has been reported in culturing MZEs of oil palm. Chehmalee and Te-chato (2008) reported that SE, SSE (80%) and completed plantlet (3.7%) can be obtained from the cross while the cross 174 (D) × 206 (P) and the cross 366 (D) × 110 (P) provided embryogenic callus (26.03%) and callus formation (26.03%) only, respectively. The highest percentage of embryogenic callus (15.8%) was found in the cross number 16 [derived from 1514 (D) × 38 (P)] (Sanputawong and Te-chato, 2008) Plantlets obtained from this procedure should be an elite hybrid suit for propagation in the field. Vigor of the seeds is generally assessed by germination test and demonstrated as germination speed index (GSI). Normally, vigor seed or zygotic embryo also has a huge silence power for germination and other activity include embryogenic callus induction (Sanputawong and Te-chato, 2008).

Somatic embryogenesis (SE) is the developmental pathway by which somatic cells develop into structures that resemble zygotic embryos (i.e., bipolar and without vascular connection to the parental tissue) through an orderly series of characteristic embryological stages without fusion of gametes (Jimnez, 2001). Somatic embryos can either differentiate directly from the explant without any intervening callus phase or indirectly after a callus phase (Wilde *et al*., 1995). The distinction between direct and indirect SE is direct SE should take place from embryogenic pre-determined cells. In contrast, indirect SE should take place from undetermined cells and an undifferentiated callus should first be formed. SE has been traditionally divided in two main stages, namely induction and expression. In the former one, somatic cells acquire embryogenic characteristics by means of a complete reorganization of the cellular state, including physiology, metabolism and gene expression (Feher *et al*., 2003). It is usually after a change in culture conditions that the induced tissues or cells reach the expression stage, in which cells display their embryogenic competence and differentiate

into somatic embryos (Suprasanna and Bapat, 2005). There are discrete developmental phases in somatic embryogenesis that are characterized by distinct biochemical and molecular events (Henry *et al*., 1994; Kawahara and Komamine, 1995; Dong and Dunstan, 2000). SE has been developed for micropropagating elite oil palm selections and for genetic manipulation using somatic cell genetic approaches. Studies were initiated by Unilever Plantations (UK), Harrison and Cross fields Plantations group (Malaysia) (Corley *et al*., 1977) and IRHO/ORSTOM in France (Rival and Parveez, 2005), and were intended to complement in-house breeding strategies for multiplying elite germplasm for commercial use. Due to the important commercial applications of the results, only limited technical information was published (Rival and Parveez, 2005). Later, several teams in Thailand developed large-scale research programmes. The first pioneer used young leaves derived from cultured zygotic embryo of oil palm to induce SE by Te-chato *et al.* (1998a). This result has revealed that the calluses could be further induced to form somatic embryos in embryogenic callus induction medium but regeneration rate of completed plantlet was quite low. Moreover, somatic embryos produced from embryogenic suspensions did not develop complete plantlet (Aberlenc-Bertossi *et al*., 1999). Te-chato *et al*. (1998a) studied on callus induction from cultured zygotic embryos of oil palm subsequent to plantlet regeneration. This result has revealed that SE was promoted by transferring to the medium containing 0.1 mg/l dicamba and culturing under a high light intensity of 4,500-6,000 lux. However, percentage and number of new forming embryos were limited and germination of those embryos was still low frequency. Te-chato *et al.* (2003) suggested that somatic embryos induced from young leaf of oil palm ortet is important material for oil palm propagation and dicamba has been reported to be an effective auxin for increasing a large number of somatic embryos. Although they could be induced directly or indirectly by the use of dicamba (Te-chato *et al*., 2003), the conversion rate of somatic embryos to plantlets is quite low. Hilae *et al.* (2005) studied on effect of carbon sources and strength of MS medium on germination of somatic embryos of oil palm. This result found that the highest percentage of shoot formation was obtained in 0.2 M sorbitol containing MS

medium and excised those shoot was cultured on root induction medium supplemented with 0.2 M sucrose promoted root formation at 31.25%.

Using zygotic embryo development as a reference, physiological and biochemical characteristics of somatic embryo maturation has been studied. Changes in several key molecules involved in acquisition of desiccation tolerance, i.e. oligosaccharides and abscisic acid (ABA), and in vigor of regenerated plantlets (such as storage proteins) have been investigated. As a result, culture conditions have been modified to enhance somatic embryo maturation (Rajesh *et al.*, 2003). Chehmalee and Te-chato (2008) success induction of somatic embryogenesis and plantlet regeneration from cultured zygotic embryo of oil palm. This result has been revealed that the plantlet conversion rate at 3.7%. Rajesh *et al.* (2003) found that putrescine containing medium could promoted SE from embryo-derived callus. However, the establishment of plant regeneration in oil palm by somatic embryogenesis is satisfactory through zygotic embryo (ZE) culture (Te-chato, 1998a; Kanchanapoom and Domyoas, 1999). It has been known that SE has been observed to be induced indirectly by different factors such as genotype, endogenous plant hormones, exogenous plant growth regulators (PGRs), and source of explant. Among those genotype is the one of importance factor influencing SE. Chehmalee and Te-chato (2007) studied that crossing among various genotypes and physiological ages of ZEs were investigated for their effect on germination or callus formation. This result revealed that both genotypes and various stages of ZEs used significantly different affected the germination and callus induction after 3 months of culture. Chehmalee (2009) found that only the cross 865 (D) × 110 (P) could provided SE, SSE and completed plantlet of oil palm while the cross 366 (D) × 110 (P) provided callus formation only. Sanputavong and Te-chato (2008) also found that different genotypes gave the different response on the percentage of cultures producing callus, embryogenic callus and number of embryogenic callus per explant. This result revealed that the highest percentage of callus formation (33.33) was obtained from cross number 7 [derived from 1514 (D) × 521 (P)] and percentage of embryogenic callus formation (18) was obtained from cross number 14 [derived from 12 (D) × 38 (P)].

The highest number of embryogenic callus formation per explant (15.795) was obtained from cross number 16 [derived from 1514 (D) × 38 (P)] after 3 months of culture, significant difference to others crosses. From the success of both mature and immature zygotic embryo it is of great important in SE of hybrid oil palm from parents of elite dura and pisifera (DxP) crosses though tissue culture technique. However, those studies have not been compared among genotypes or cross combination from MZEs of hybrids oil palm.

The objective of the present study is: to investigate the effect of hybrid oil palm's genotypes on embryogenic callus formation from MZEs, 2) Average number of embryogenic callus formation, 3) the effect of genotype on percentage, average number and type of somatic embryogenesis.

Materials and Methods

Plant material

Sterilized HMZEs of oil palm from six genotypes, cross #58, cross #77, cross #118, cross #119, cross #130 and cross #137 were inoculated in culture tubes (25×150 mm) containing 10–15 ml of modified MS (Murashige and Skoog) medium supplemented with 2.5 mg/l dicamba. These culture media was adjusted pH to 5.7 with 0.1 N KOH before adding 0.75% agar and autoclaving at 1.05 kg/cm^2, 121°C for 15 min. The cultures were placed under light conditions of 40 μmol/m^2/s for 16 h photoperiod at 25±2°C and subcultured every 4 weeks on the same medium component for 3 months.

Effect of genotypes on percentage and average number of embryogenic callus formation

Calluses induced from HMZEs (Half mature zygotic embryos) of cross #58, cross #77, cross #118, cross #119, cross #130 and cross #137, were regularly transferred to fresh MS medium monthly interval. The medium was supplemented with 1 mg/l dicamba, 200 mg/l ascorbic acid, 3% sucrose and gelled with 0.75% agar-agar

(Aslan, 2008). Each experiment was performed with 4 replicates. Each replicate consisted of 10 test tubes (25×150 mm containing 10 ml of medium). The pH of medium was adjusted to 5.7 prior autoclaving at 1.05 kg/cm^2 at 121^{o}C for 15 min. Cultures were maintained at 28±0.5ºC under 14 h photoperiod at 3,000 lux illumination and subcultured monthly intervals. The percentage of embryogenic callus formation and number of embryogenic callus formation were recorded after 1 month for 3 months.

Effect of genotypes on percentage, average number and type of somatic embryogenesis

Primary callus (PC) induced from HMZEs of six crosses; cross #58, cross #77, cross #118, cross #119, cross #130 and cross #137, according to was regularly transferred to fresh MS medium monthly interval. The medium was supplemented with 1 mg/l dicamba, 200 mg/l ascorbic acid, 3% sucrose and gelled with 0.75% agar-agar. Each experiment was performed with 4 replicates. Each replicate consisted of 10 test tubes (25×150 mm containing 10 ml of medium). The pH of medium was adjusted to 5.7 prior autoclaving at 1.05 kg/cm^2 at 121^{o}C for 15 min. Cultures were maintained at 28±0.5ºC under 14 h photoperiod at 3,000 lux illumination and subcultured monthly intervals. The percentage of somatic embryo formation and type of somatic embryo formation were recorded after 3 months of culture.

Data analyses

For experimental design and statistical analysis, completely randomized design (CRD) with 4 replicates (each replicate consist of 10 embryos) was performed. The percentage of cultures that produced embryogenic callus, number of the embryos and speed of embryogenic callus index (SECI) were recorded and compared among those crosses every month for 3 months. The speed of germination index was calculated and modified according to Santipracha (2002). The average number of embryogenic callus was assessed each explants. The percentage of cultures that produced somatic

embryo and type of somatic embryo were recorded and compared among those crosses. Data were analyzed using analysis of variance (ANOVA version 14.0). Means were separated by Duncan's multiple rang tests (DMRT) at 0.05 level of probability.

- Speed of embryogenic callus induction index (SECI) was calculated as below;

$$= \frac{\text{Explant with embryogenic } 1^{st}}{1} + \frac{\text{Explant with embryogenic } 2^{nd}}{2} + \frac{\text{Explant with embryogenic } 3^{rd}}{3} + \ldots + \frac{\text{Explant with on final day}}{n}$$

Results

Effect of genotypes on percentage and average number of embryogenic callus formation

After 6 weeks of subculture onto MS medium supplemented with 1 mg/l dicamda and 200 mg/l ascorbic acid, all genotypes were promoted to form embryogenic callus form MZEs. Two main types of calluses were observed in cultures; non-embryogenic callus (NEC) and embryogenic callus (EC). NEC callus were characterized by a cream color and a soft, loose, watery nature. EC were yellow or pale yellow in color, compact and contained embryonic structures occurring either as independent or fused nodules. The distinctive types of culture were more apparent after the second transfer onto culture media. The analysis of variance (ANOVA) showed that the genotypes and phytohormone played significantly role on the percentage of embryogenic callus after 3 months of culture. Cross #58 was identified as the best genotype and produced the highest percentage of embryogenic callus (50.65), significantly different to other genotypes after 3 months of culture. Besides, cross combination played important role on average number of embryogenic callus formation after 3 months of culture. The result also revealed that cross #58 gave the highest

average number of embryognic callus (9.14), followed by cross 137# (8.23) while cross #118 gave the lowest average number of embryognic callus (3.84) after 3 months of culture (Table 2, Figure 7).

For speed of embryogenic callus index (SECI), it was showed that cross #58 gave the highest result at 11.72, followed by cross 137# (7.94) and cross #77 (6.78), respectively (Figure 8). It is indicated that cross #58 had the hybrid vigor more than another crosses.

Table 2 Effect of genotypes on embryogenic callus induction of hybrids oil palm after cultured on MS medium supplemented with 1 mg/l dicamba and 200 mg/l ascorbic acid and 3% sucrose for 3 months

Crosses	Embryogenic callus (%)	Average number of embryogenic callus/explant
Cross #58	50.65a	9.14a
Cross #77	37.08b	7.24ab
Cross #118	26.77d	3.84c
Cross #119	28.25d	5.86b
Cross #130	26.38d	7.34ab
Cross #137	33.33c	8.23ab
F-test	*	*
C. V. (%)	23.56	33.40

*Significant different at $\rho < 0.05$

Mean followed by the same letter within column do not significantly different at 5% level by DMRT

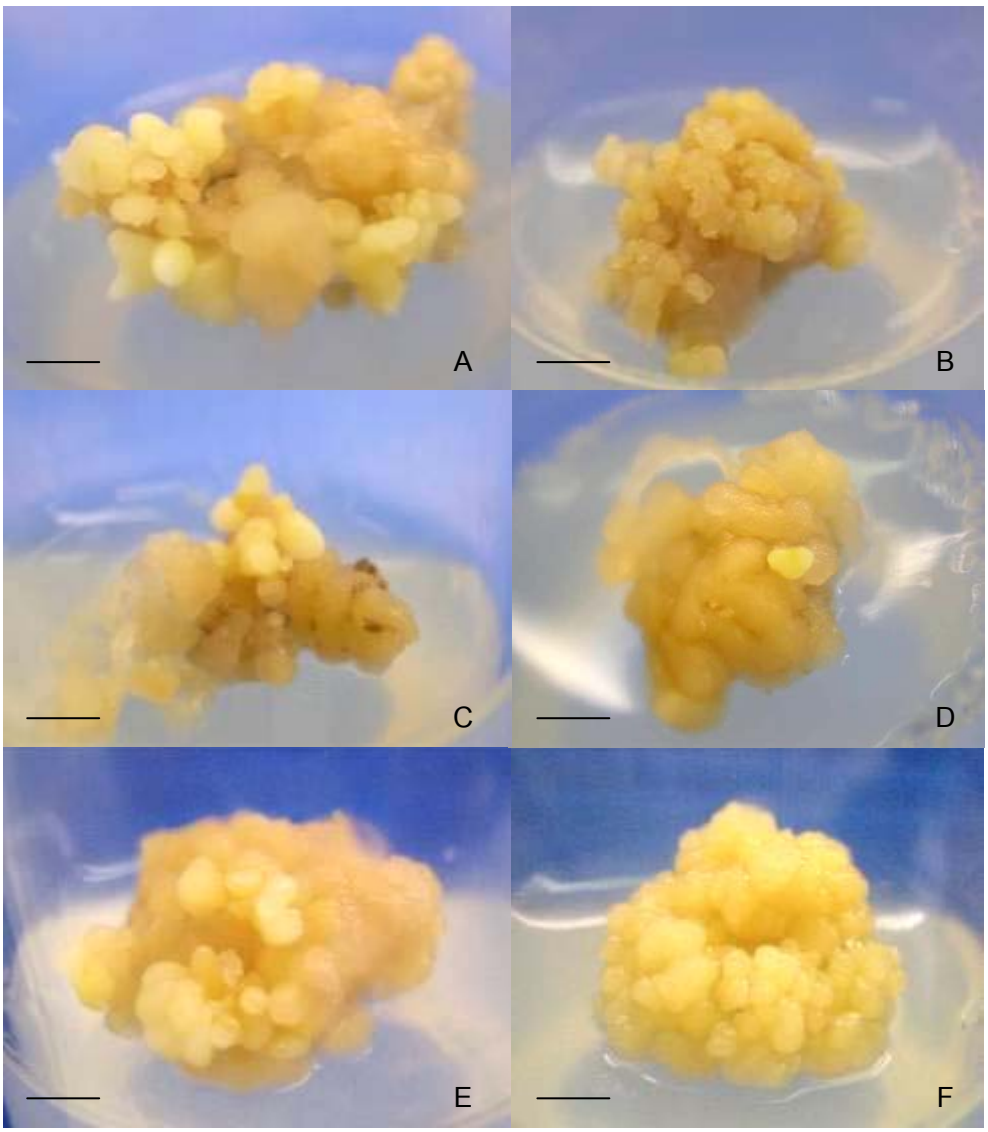


Figure 7 Embryogenic callus formation of mature zygotic embryo from cross #58 (A), cross #77 (B), cross #118 (C), cross #119 (D), cross #130 (E) and cross #137 (F) cultured on solid MS supplemented with 1 mg/l dicamba and 200 mg/l ascorbic acid for 3 months (bar = 2.50 mm).

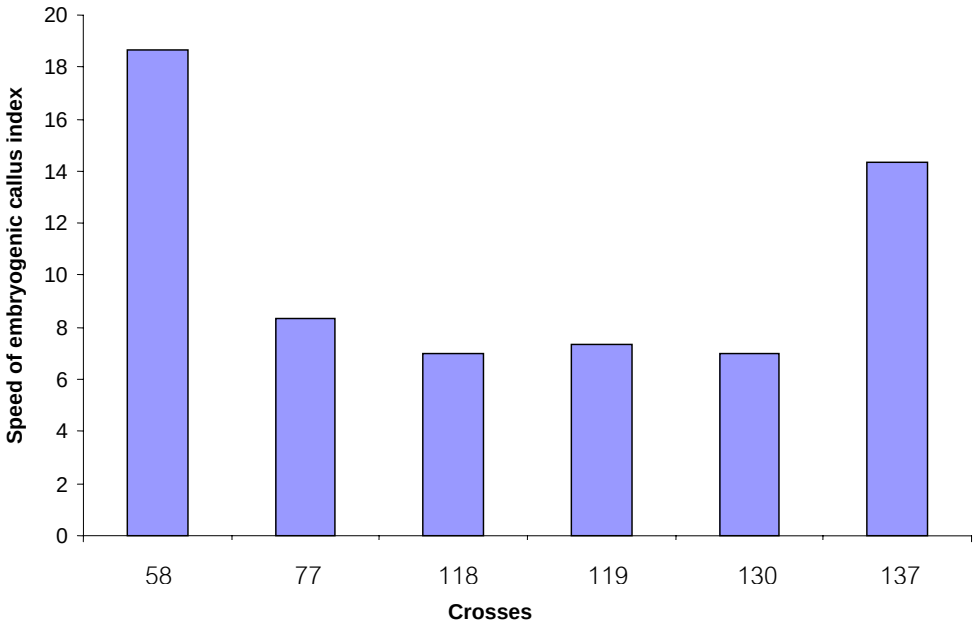


Figure 8 Effect of genotypes on SECI on MS medium supplemented with 1 mg/l dicamba and 200 mg/l ascorbic acid after 3 months of culture.

Effect of genotypes on percentage, average number and type of somatic embryogenesis

PC of six crosses was derived from HMZEs of hybrids oil palm on MS medium supplemented with 2.5 mg/l dicamba after 3 months in culture. The embryogenic culture was induced when PC were transfer to MS medium supplemented with 1 mg/l dicamba and 200 mg/l ascorbic acid. The cultures embryogenic callus could be maintained for more than 6 weeks. Regeneration of somatic embryogenesis was initiated after 2 weeks of culture on MS supplemented with 1 mg/l dicamba and 200 mg/l ascorbic acid. All genotypes were promoted to form somatic embryos form embryogenic callus (Figure 9). Proembryogenic masses (PEM) differentiated into proembryos and globular structures grew out from callus being visible on its surface. Some globular structures were able to develop hasutorium structures after 14 days of culture. Therefore, the present studies found that these somatic embryos could be classified into

two main stages; globular-staged embryos (GS) and haustorium-stage embryos (HS). GS were characterized by a cream or pale cream in color and a soft, loose, watery nature (Figure 9A). HS were greenish color and compact textures (Figure 9D). The analysis of variance showed that genotypes and hormone used significantly different affected the percentage of somatic callus and average number of somatic callus formation after 3 months of culture. Cross #58 was identified as the best genotype which produced the highest percentage of somatic embryo at GS (17.32), significantly different other genotypes after 3 months of culture. The result also revealed that cross #58 gave the highest HS at 7.92%, followed by cross #130 and cross #137which provided HS at 6.90 and 5.35%, respectively. While cross #118 gave the lowest somatic embryo of HS at 1.11% after 3 months of culture (Table 3).

For average number of somatic embryo, cross #58 gave the highest average number of GEs at 13.38 ± 3.65, significantly different other genotypes after 3 months of culture. The result also revealed that cross #58 gave the highest average number of HEs at 4.00 ± 1.33, followed by cross #77 and cross #137 which provided average number of HEs at 2.98 ± 0.78 and 2.80 ± 1.22, respectively. While cross #118 gave the lowest average number of HEs at 0.20 ± 0.03 after 3 months of culture (Table 3).

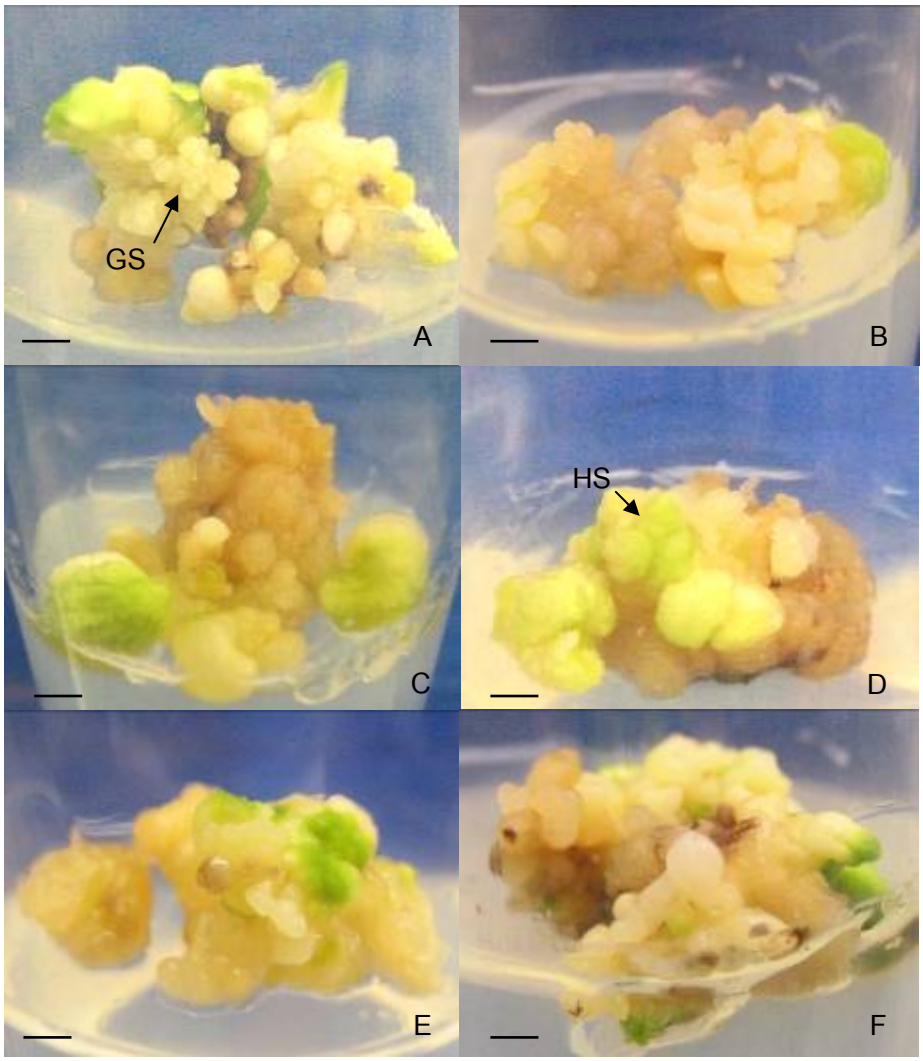


Figure 9 Type of embryogenic callus developed from culturing MZEs of cross #58 (A), cross #77 (B), cross #118 (C), cross #119 (D), cross #130 (E) and cross #137 (F) on solidified MS medium supplemented with 1 mg/l dicamba and 200 mg/l ascorbic acid for 3 months (bar = 2.50 mm).

Table 3 Effect of genotype on the percentage and average number of somatic embryos of hybrids oil palm after culture on MS supplemented with 1 mg/l dicamba and 200 mg/l ascorbic acid for 3 months

Crosses	Somatic embryos (%)		Average number of somatic embryo / explant	
	GS	HS	Globular ± SD	Haustorium ± SD
Cross #58	17.32a	7.92a	13.38 ± 3.65a	4.00 ± 1.33a
Cross #77	7.17d	4.59b	7.33 ± 2.71b	2.98 ± 0.78b
Cross #118	2.14d	1.25b	0.66 ± 0.05d	0.20 ± 0.03c
Cross #119	2.16d	1.11b	1.67 ± 2.38b	0.67 ± 0.08c
Cross #130	11.31b	6.90a	9.92 ± 6.55b	2.50 ± 1.58b
Cross #137	8.69c	5.35ab	6.00 ± 1.73c	2.80 ± 1.22b
F-test	*	*	*	*
C.V. (%)	22.45	26.78	35.62	37.77

* Significant different at $\rho < 0.05$

Mean followed by the same letter within column do not significantly different at 5% level by DMRT

Discussion

Effect of genotypes on percentage and average number of embryogenic callus formation

Embrygenic callus can be considered as the expression of the totipotentiality of plant cells and it is well established that the expression of this feature is controlled by genetic factor (Ezhova, 2003). In the present study, a significant influence of the genotype on the percentage of embryogenic callus formation and average number of embryogenic callus were observed. Significantly difference was found among genotypes, cross #58 had the best percentage of embryogenic callus formation and average number of embryogenic callus. The differences in the embryogenic callus formation of the genotypes might be depended on genetic make up of each parents and growing conditions of the donor plants. Effects of genotype on embryogenic callus formation were observed in many plants such as date palm (*Phoenix dactylifera* L.) (Al-Khayri and Al-Bahrany, 2004), peach palm (*Bactris gasipaes* Kunth) (Steinmacher *et al*., 2007) and on the development of friable embrygenic callus from immature inflorescence of African oil palm (Teixeira *et al*., 1995). Chehmalee and Te-chato (2008) studied in oil palm hybrids and reported different resulted from different cross. Embryogenic callus was obtained from the cross 174 (D) × 206 (P) while only callus was formed in the cross 366 (D) × 110 (P). The same finding was reported in 16 crosses of oil palm by Sanputawong and Te-chato (2008). Cross number 16 [derived from 1514 (D) × 38 (P)] produced the highest percentage of embryogenic callus formation (15.80%). Khanna and Raina (1998) concluded that the genotypes influenced differently both on callus and embrogenic callus formation. Similar results were also reported by Seraj *et al.* (1997) who studied in rice.

However, no genotype effect on embryogenic callus formation from coconut inflorescence (Verdeil *et al*., 1994). This result suggested that it might be depended on source and endogenous of explant. Feher *et al.* (2003) reviewed that the choice of explant can in some species be of importance for obtaining embryogenic cell-

lines. Plant species which are capable of expressing their embryogenic potential regardless of the explant include *Daucus carota* and *Medicago sativa*, while for many other species embryonic or highly juvenile tissue has to be used as the explant (Gall-Meagher and Green, 2002). Litz and Gray (1995) reported that the pattern of the developmental response of cultured tissue is epigenetically determined and is influenced by genotype, the stage of development of the plant and the nature of the explant. Mature embryo is regarded as the most efficient tissue source for regenerating whole plants in large number but the response is very genotype specific (Elena and Ginzo, 1988; Feher *et al*., 2003). Ozgen *et al*. (1998) observed that a high genotypic influence on embryogenic callus induction from immature and mature embryos of winter wheat (*Triticum aestivum* L.). Miguel and Walter (1998) also found that using zygotic embryo of *E. edulis* as explants gave the highest embryogenic callus formation than using immature inflorescence and young leaf. Khaleda and Al-Forkan (2006) demonstrated that mature seed scutellum is the best explants among the explants for highly totipotent embryogenic callus initiation.

Vigor of the seeds is generally assessed by germination test and demonstrated as germination speed index (GSI). Normally, vigor seed or zygotic embryo also has a huge silence power for germination and other activity include callus or embryogenic callus formation. For SECFI in this study, cross #58 gave the highest result (11.72). It is suggested cross #58 had the highest vigor more than other crosses. Similarity results was obtained by Sanputawong and Te-chato (2008) who found that cross number 16 had not only the highest vigor but also the highest SECFI (20.17). The reason could be due to the good combining of gene between the two parents (Sanputawong and Te-chato, 2008).

Effect of genotypes on percentage, average number and type of somatic embryogenesis

In most somatic embryogenesis, embryogenic potential is induced soon after the explant are exposed to PGRs. Mostly auxins such as 2,4-D, NAA and dicamba were used in oil palm. Beside, characteristic embryogenic or highly juvenile tissue types are most appropriate material for somatic embryogenesis. The number of cells that are able to exhibit embryogenic potential is limited and this fraction is highly variable among plant species. It depends on the genotype (Feher *et al*., 2003). The result from the present study showed that there were significantly different of genotypes on somatic callus formation and average number of somatic callus formation. Cross #58 was identified as the best strong genotype and produced the highest percentage of somatic embryo, 17.32% of GS and 7.92% of HS were obtained when cultured on MS medium supplemented with 1 mg/l dicamba and 200 mg/l ascorbic acid. Similar result to Chehmalee and Te-chato (2008), they reported that different genotypes or cross combination gave the different response on types of SE induction. This result revealed that cross 174(D) × 206(P) gave the highest GS formation (26.03%) which contained many small pearly white globular structure followed by 366(D) × 110(P) and 865(D) × 110(P), respectively. In case of HS development, both 865(D) × 110(P) (14%) and 174(D) × 206(P) (10%) produced greenish color of HS which separate as individual embryo whereas cross 366(D) × 110(P) fail to produce HS. Similar result was obtained in culturing immature inflorescence of peach palm (Steinmacher *et al.*, 2007). Different varieties resulted in the different response on SE formation and plantlets development (Karun *et al*., 2004). Sarasan *et al*. (2005) also reported that genotype of the selected explants have influenced upon the type and percentage of callus formation. In comparison of seed vigor, the larger seeds consisted of larger size of ZE of all crosses gave the higher percentage of germination and callus formation (Chehmalee and Techato, 2007). Chehmalee (2009) also found that only the cross 865 (D) × 110 (P) provided SE, SSE and completed plantlets of oil palm while the cross 366 (D) × 110 (P) provided callus only. It is clear evidence that genotype play role in SE and endogenous

hormone metabolism. Feher *et al.* (2003) reported that continual culture of non-embryogenic callus in the same concentration of auxin containing medium inhibited the development of embryogenic cells. Reduction of auxin in subculturing medium has been triggered somatic embrygenesis. These observations indicated a considerable difference between the auxin sensitivity of the two genotypes. The key role of endogenous hormone metabolism affected by genetic, physiological and culture environments are well accepted in the induction phase of somatic embryogenesis (Jime, 2001). Sanputavong and Te-chato (2008) also found that different genotypes gave the different response in the percentage of cultures producing callus, embryogenic callus and number of embryogenic callus per explant. The reason could be due to the good combining ability of gene between the two parents.

The result of the present study showed that cross #58 was identified as the best genotype. Therefore, it is possible that this genotype might be from the good combination between the two parents. SE was induced in all genotypes tested, even though it was possible to detect a genotype-specific response both in terms of the frequency of SE as well as in the average number of somatic embryos per explant. Cross #58 gave the highest percentage average number of somatic embryo at GEs (13.38 ± 3.65) and HEs (4.00 ± 1.33) when cultured on MS medium supplemented with 1 mg/l dicamba and 200 mg/l ascorbic acid after 3 months of culture. SE in this result could be induced from all genotypes tested which have the genetic potential to form embryos from somatic cells. However, cross #118 and cross #119 provided both percentage and average number of SE less than other crosses even though both crosses obtained from the same parents of pisifera are 206 (P). So, those crosses gave the lowest capable of SE formation. This evidence may be due to influence of the parental genetic. In most plant species, embryogenic competence or capable of SE is restricted to ability tissues of a given genotype (Feher *et al*., 2003).

Several authors have reported that specific modifications of culture medium, such as the addition of auxins, greatly influence somatic embryogenesis frequency in oil palm (Aberlence-Bertossi *et al*., 1999; Teixeira *et al*., 1994; Teixeira *et*

al., 1995). Dicamba, one of effective synthetic auxins was used, and has been reported to increase a large number of somatic embryos (SEs) (Te-chato *et al*., 2003). This result has revealed that the highest percentage of somatic embryo was obtained in 0.1 mg/l dicamba containing MS medium. Contrary to this study, the addition of 0.1 mg/l dicamba to the induction medium inhibited growth of embryogenic callus (data not shown). The different response might be due to the different source of explant used for callus induction. Similar results in buffel grass (Colomba *et al*., 2005) and forage grass (Denchev and Conger, 1995). Explants, such as immature and mature embryos, and young leaf, have been used to initiate embryogenic callus cultures capable of plant regeneration in oil palm. Among those explants, mature seed are preferred over other explants because they lack seasonal restriction on availability and can be used directly in tissue culture, thus avoiding the laborious isolation of immature tissues (Zale *et al*., 2004). Previous studies on oil palm tissue culture have demonstrated in plant regeneration from embryogenic callus induced from young leaf (Te-chato *et al*., 2003) and young inflorescence tissues (Teixeira *et al*., 1995). However, genotypes of the selected explants may have influenced upon the type of responsive callus and embryogenic callus, as reported in the former chapter 3.1 and 3.2. The same result was also reported by Sarasan *et al*. (2005) and Sanputavong and Te-chato (2008).

CHAPTER 3

Propagation of Hybrid Oil Palms by Culturing of Zygotic Embryos

Experiment III

Effect of genotype on the percentage, average number of secondary somatic embryos and plantlet regeneration

CHAPTER 3

EXPERIMENT III

Introduction

Secondary somatic embryogenesis (SSE) is a phenomenon whereby new somatic embryos are initiated from somatic embryos (Vasic *et al*., 2001). It has, compared to primary somatic embryogenesis, advantages such as a high multiplication rate, independence of an explant source and repeatability. Somatic embryos have shown to be excellent source for secondary embryos. This is associated with loss of integrated group control of cell organization in the somatic embryos. Some cells break away from group control and initiate new somatic embryos. In many species immature zygotic embryos possess and mature zygotic embryos lack the ability to express somatic embryogenesis. Furthermore, embryogenicity can be maintained for prolonged periods of time by repeated cycles of secondary embryogenesis (Raemakers *et al*., 1995). In many species the efficiency of explants in primary embryogenesis is lower than in secondary embryogenesis. This phenomenon has been described in at least 80 Gymnosperm and Angiosperm species (Raemakers *et al*., 1995). High-frequency plant regeneration systems through secondary embryogenesis were reported in several plant species of interest (Merkle *et al.,* 1990; Raemakers *et al*., 1993a,b; Weissinger II and Parrott, 1993; Choi *et al*., 1997; Das *et al*., 1997; das Neves *et al*., 1999; Chen and Chang, 2004; Giridhar *et al*., 2004). SSE in oil palm has been documented from young leaf (Te-chato *et al*., 2007) and zygotic embryo derived callus (Nwanko and Krikorian, 1983; Rabechault and Cas, 1974). However, the establishment of plant regeneration in oil palm by somatic embryogenesis is satisfactory. Many factors influence the efficiency of a regeneration procedure using SSE. The main factors determining the tissue culture response in oil palm include genotype, donor plant and culture medium. An in-depth study of such factors would enable the development of genotype-specific culture

methods to better enhance the tissue culture response of oil palm. The ability of oil palm zygotic embryos to produce secondary embryos has also been indicated (Rajesh *et al.,* 2003). However, percentage and numbers of new forming embryos were limited and germination of those embryos was not reported. Te-chato and Hilae (2007) reported that full-strength MS medium supplemented with 0.2 M sorbitol produced SSE from haustorium embryo (HE) derived from culturing young leaf of mature oil palm subsequent to a high frequency of plantlet formation. Chemalee and Te-chato (2008) also found that formation of SSE from ZE-derived HE subsequent to plantlet regeneration when cultured on MS medium supplemented with 0.2 M sorbitol and 200 mg/l ascorbic acid after 3 months of culture. However, there have no reports on genotypes or cross combination play role on SSE formation from HMZEs culture.

The objectives of the present study was to investigate the effect of genotypes on the percentage of SSE, average number of SSE formation and germinated plantlet derived from primary haustorium embryos (PHEs) of hybrid oil palms.

Materials and Methods

Plant materials

Sterilized HMZEs of oil palm from six genotypes, cross #58, cross #77, cross #118, cross #119, cross #130 and cross #137 were inoculated in culture tubes (25×150 mm) containing 10–15 ml of modified MS (Murashige and Skoog) medium supplemented with 2.5 mg/l dicamba. These culture media was adjusted pH to 5.7 with 0.1 N KOH before adding 0.75% agar and autoclaving at 1.05 kg/cm^2, 121°C for 15 min. The cultures were placed under light conditions of 40 μmol/m^2/s for 16 h photoperiod at 25±2°C and subcultured 4-week or monthly intervals on the same medium component for 3 months.

Effect of genotypes on the percentage and average number of SSEs

PHEs induced from HMZEs of six crosses; cross #58, cross #77, cross #118, cross #119, cross #130 and cross #137 were carefully detached and were cultured on fresh MS medium. The medium was supplemented with 0.2 M sorbitol, 200 mg/l ascorbic acid, 3% sucrose and gelled with 0.75% agar-agar. Each experiment was performed with 4 replicates. Each replicate consisted of 10 test tubes (25×150 mm containing 10 ml of medium). The pH of medium was adjusted to 5.7 prior autoclaving at 1.05 kg/cm^2 at 121^{o}C for 15 min. The cultures were maintained at 28±0.5ºC under 14 h photoperiod at 3,000 lux illumination. The percentage of SSE formation and average number of SSE formation were recorded after 4 months of culture.

Effect of genotype on the germination percentage of SSE

SSE together with PHEs of six crosses; cross #58, cross #77, cross #118, cross #119, cross #130 and cross #137 were carefully transferred to cultured on fresh MS medium. The medium was supplemented with 200 mg/l ascorbic acid, 3% sucrose and gelled with 0.75% agar-agar (Aslan, 2008). The pH of medium was adjusted to 5.7 prior to autoclaving at 1.05 kg/cm^2 at 121^{o}C for 15 min. Each experiment was performed with 4 replicates. All cultures were carried out in 25x150 mm culture bottle under 14 h photoperiod, 1,300 lux illumination. After 4 months (observed monthly interval) germination percentage and number of seedlings/SSE clump were recorded.

Data analyses

For experimental design and statistical analysis, completely randomized design (CRD) with 4 replicates (each replicate consist of 10 embryos) was performed. The percentage of cultures that produced SSE, average number of SSE, germination percentage and number of seedlings/SSE clump were recorded and compared among those crosses. Data were analyzed using analysis of variance (ANOVA).

Results

Effect of genotypes on the percentage and average number of SSEs

PHEs of six crosses were derived from HMZEs of hybrids oil palm on MS medium supplemented with 1 mg/l dicamba and 200 mg/l ascorbic acid after 2 months in culture. All genotypes promoted SSE formation form PHEs when cultured on MS medium supplemented with 0.2 M sorbitol and 200 mg/l ascorbic acid for 4 months. SSE was induced from the basal part of PHEs. Those SSE were cluster with white opaque characteristic consisted of torpedo-stage embryos (Figure 10). The analysis of variance showed that genotype and PGR played significant role in the percentage of SSE formation and average number of SSEs after 4 months of culture. Cross #58 was identified as the best genotype and produced the highest percentage of SSE (36), significantly different other genotypes after 3 months of culture. The result also revealed that cross #58 gave the highest average number of SSE at 21.56 ± 14.22, followed by cross #137 and cross #130 which provided average number of SSE at 15.92 ± 9.56 and 11.55 ± 8.98, respectively. While cross #118 and cross #119 could not promoted SSE formation after 4 months of culture (Table 5).

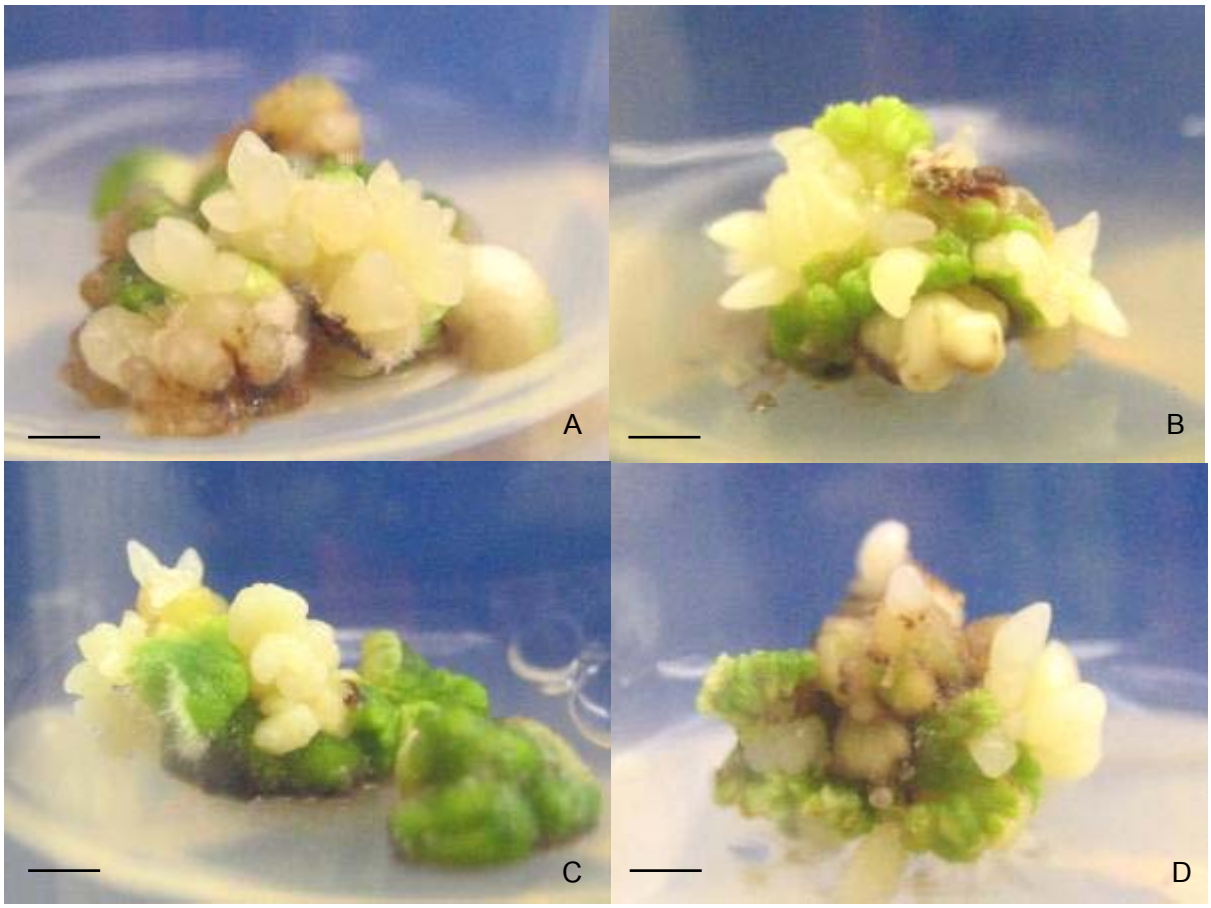


Figure 10 SSE formed from PHEs of MZEs from cross #58 (A), cross #77 (B), cross #130 (C) and cross #137 (D) cultured on solid MS supplemented with 0.2 M sorbitol and 200 mg/l ascorbic acid for 4 months (bar = 2.50 mm).

Table 4 Effect of genotype on the percentage of SSE formation and average number of SSEs after culture on MS supplemented with 0.2 M sorbitol and 200 mg/l ascorbic acid for 4 months.

Crosses	SSE formation (%)	Average no. SSE / PHE SSE ± SD
Cross #58	36a	21.56 ± 14.22a
Cross #77	7c	6.60 ± 3.77c
Cross #118	0d	0.00 ± 0.00d
Cross #119	0d	0.00 ± 0.00d
Cross #130	18b	11.55 ± 8.98b
Cross #137	15b	15.92 ± 9.56b
F-test	*	*
C.V. (%)	13.88	24.42

*Significant different at $p \leq 0.05$

Mean followed by the same letter within column do not significantly different at 5% level by DMRT

Effect of genotype on the germination percentage of SSE

SSE formed from the basal part of PHE (Figure 11A) after 3 months of transfer onto stress medium (MS supplemented with 0.2 M sorbitol and 200 mg/l ascorbic acid. Some of those SSEs developed into mature SSE with green shoots (Figure 11B). Germination of shoots (Figure 11C) and complete plantlets (Figure 11D) were observed just after one month of transfer onto PGR-free MS medium.

Almost genotypes could be developed completed plantlets form SSE when cultured on hormone-free MS medium supplemented with 200 mg/l ascorbic acid. Three main types of growth responses were observed: SSE producing shoot (Figure 12A), root (Figure 12B) and both shoot and root (Figure 12C). The distinctive types of culture were more apparent after the second transfer onto culture media. After 3 months of subculture on various kinds of medium, healthy and normal seedlings of some genotypes were obtained (Figure 13). Significantly different on the percentage of germinated plantlet from SSE after 3 months of culture among genotypes were observed. Cross #58 was produced the highest percentage of completed plantlets (38.57%) while 27.27% produced only shoot and 18.18% produced only root after 3 months of culture. No SSE formation was observed from cross #118 and cross #119 after 3 month of culture (Table 6).

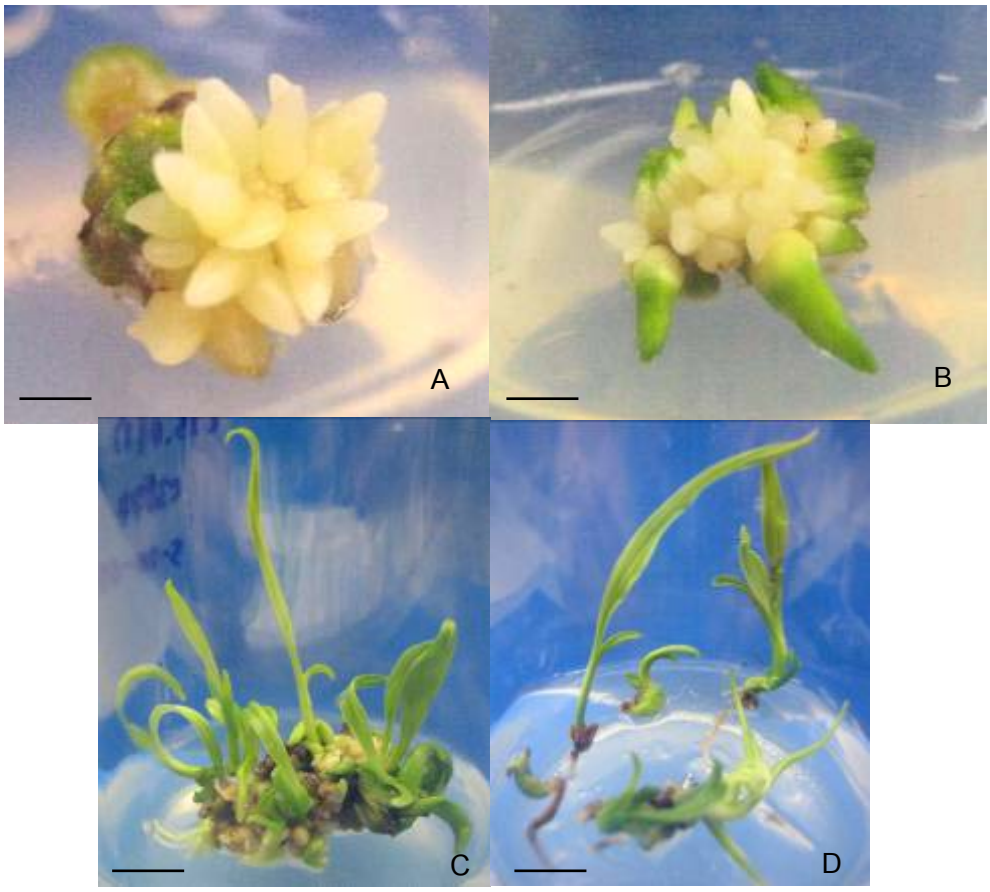


Figure 11 Development of SSE from basal part of PHEs (A) on 0.2 M sorbitol containing MS medium for 3 months subsequent to maturity of SSEs (B), germination of shoots (C) and complete plantlets (D) on PGR-free MS medium for further 1 | months

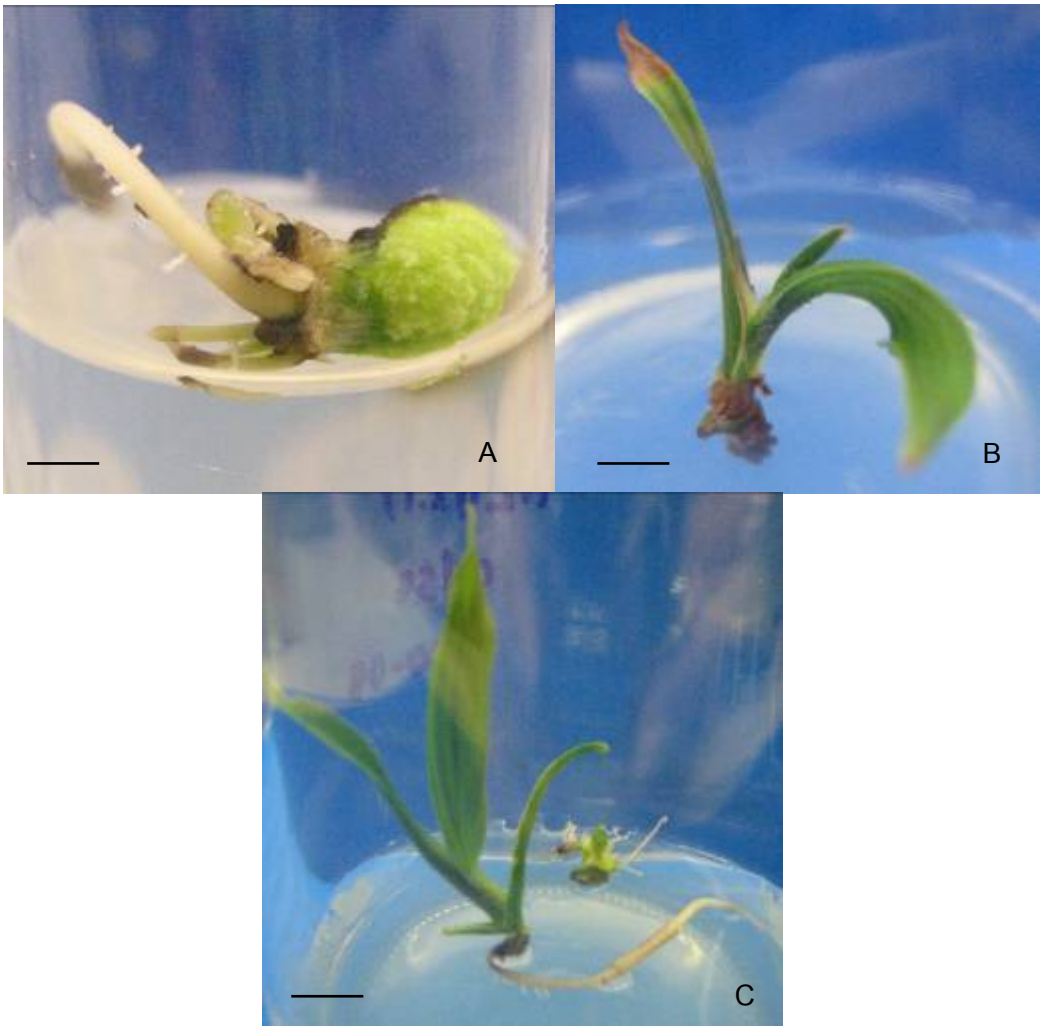


Figure 12 Various types of growth response from SSE on hormone-free containing MS medium supplemented with 200 mg/l ascorbic acid for 1 to 3 months.

A: root only

B: shoot only

C: both shoot and root

Table 5 Effect of genotype on percentage of completed plantlet on hormone-free MS medium supplemented with 200 mg/l ascorbic acid and 3% sucrose for 3 months.

Crosses	SSE producing shoot (%)	SSE producing root (%)	Complete plantlet (%)
Cross #58	27.27a	18.18a	38.57a
Cross #77	12.74b	5.44b	22.33b
Cross #118	0.00c	0.00c	0.00c
Cross #119	0.00c	0.00c	0.00c
Cross #130	10.83b	5.32b	19.17b
Cross #137	14.58b	4.17b	25.42b
F-test	*	*	*
C.V. (%)	13.06	14.22	17.48

*Significant different at $p \leq 0.05$

Mean followed by the same letter within column do not significantly different at 5% level by DMRT

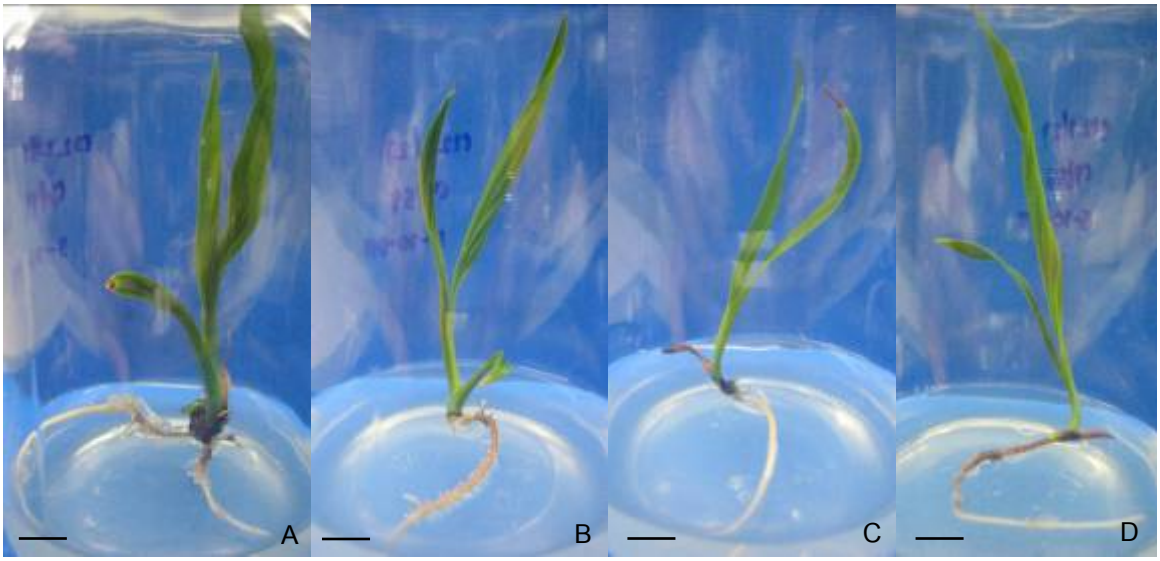


Figure 13 Complete plantlets through SSE from cross # 58 (A), cross # 77 (B), cross # 130 (C) and cross # 137 (D) on solidified PGR-free MS medium for 3 months.

Discussion

Sources of explant for induction of SSE were differed from species to species. Immature male inflorescence of banana was proved to be a good starting explant for the regeneration of plants via SSE (Khalil *et al.,* 2002). Since new embryos are continually formed from existing embryos, SSE has the potential to produce many plants and, once initiated, may continue to produce embryos over a long period of time. In oil palm, zygotic embryos were tried but small number of SSE induced (Rajesh *et al.,* 2003; Te-chato and Hilae, 2007).

In this present study, PHEs were great important for utilization in inducing SSE and there were significantly different affected of genotypes on SSE formation and average number of SSE ($p \leq 0.05$). Cross #58 gave the highest percentage of SSE (36%) and average number of SSEs (21.56 ± 14.22) when cultured on MS medium supplemented with 0.2 M sorbitol and 200 mg/l ascorbic acid, significantly different

other genotypes after 4 months of culture ($p \leq 0.05$). Those SSEs were white, opaque and torpedo in shape which arisen directly from peripheral layer of basal part of PHE like the report of Promchan and Te-chato (2007). SSEs were also reported to be induced indirectly from SE-derived cotyledon in cotton (Raemakers *et al*., 2000), cocao (*Theobroma cacao*) (Siela *et al*., 2002), madagaskar periwinkle (*Catharanthus roseus*) (Junaid *et al*., 2007) and carnation (*Dianthus caryophyllus*) (Omid *et al*., 2008). Not only genotypes play role on SSE formation but plant growth regulators and chemical factor also act as promoting SSE. From this result, it is suggested that genotype play significant role over other factors. The hybrid seeds of oil palm from elite parents, dura and pisifera (D x P) should be screened before mass scale production of F1 unique clones. From this experiment it is clearly seen that cross #58 showed a high potential for that purpose. These parents have been improved by conventional breeding program from agricultural research station. Therefore, it is possible that this genotype might be a superior genotype from the good combination ability of gene between the two parents. Contrary result was reported by Jimnez (2001) who found that the endogenous hormone level of the initial explant is essential for determining the ability of genotype to induce somatic embryogenesis. This result performed comparative investigation of two *M. falcata* lines confirms that the level of endogenous indole-3-acetic acid (IAA) in the initial explant was higher in the embryogenic line. These studies indicated that the processes of *in vitro* somatic embryogenesis are the results of a proper balance of plant growth regulators supplied to the culture medium and endogenous regulators in the tissue of the primary explant.

Moreover, the result from this present study showed SSE of some tested genotypes could be developed into complete plantlets. Cross #58 gave the highest percentage of germinated plantlet (38.57) when cultured on MS medium supplemented with 200 mg/l ascorbic acid, significantly different other genotypes after 3 months of culture ($p \leq 0.05$). Similar results were obtained in *Medicago falcata* (Chen *et al*., 1987). Genotype is the most important factor influencing plant regeneration response. Variability in the induction frequency of SSE is observed among different species and

within cultivars (Brown and Atanassov, 1985). Beside genotype there are other factors affecting plant regeneration: explant, culture condition, medium composition and PGRs. Typically, the removal of the auxin from medium suppressed newly forming embryos. However, this is not necessary in all cases. In oil palm normal germination of oil palm SE developed normal seedlings in liquid MS or ½MS mediuin in the presence of low concentration of NAA (0.06 mg/l) and benzylaminopurine (BA) (0.03 mg/l) (Techato and Muangkaewngam, 1992). However, low efficiency of germination was reported. Te-chato and Hilae (2007) modified sugars and plant growth regulators together with various strengths of MS medium. It was clear evident that SSE induced on 0.2 M sorbitol and 0.1 mg/l dicamba containing full strength MS medium could germinated plantlets at high frequency at 78%. However, conversion rate of SSE to plantlet was still low frequency at 3.7% (Chemalee and Te-chato, 2008). The main difficulty in conversion of SSE into plants appears to be that the embryos are not actually mature, a stage characterized by accumulation of embryo-specific reserve food materials and proteins and by desiccation tolerance (Te-chato and Hilae, 2007). In banana, mature SE germinates into plants after desiccation or dehydration and culture on a MS medium supplemented with BA (Khalil *et al.,* 2002). Alcohol sugar or polyol e.g. polyethylene glycol (PEG) acts as osmotic stress and has a function like desiccation has been reported affecting on maturation and synchronization of the embryos (Mamiya and Sakamoto, 2000). In this present study, PEG was not tried but sorbitol enhanced SSE formation. High levels of sucrose have been reported to enhance normal maturation in somatic embryos including the addition of mannitol (4%) to the medium but failed to promote maturation and germination in oil palm (Te-chato and Hilae, 2007). Those phenomena mimic the developmental environment of zygotic embryos and should further improve the maturation of somatic embryos (Te-chato and Hilae, 2007). In this present study it is clear evident that genotype play role in SSE induction and germination of SSE.

CHAPTER 4

Screening and detection of hybrids oil palm by DNA markers

Experiment I

Verification of hybrids oil palm by random amplified polymorphic DNA (RAPD) and simple sequence repeat (SSR) markers

CHAPTER 4

EXPERIMENT I

Introduction

Determination or verification of F1-hybrid seeds is a quality control requirement in the production of hybrids oil palm seed, to avoid unacceptable contamination with natural or artificial self-pollination of the female parent. The success of hybrid oil palm production beside other factors depends on the production and timely supply of genetically homogenous seeds to farmers. This ensures that the gains of heterosis can be harnessed through enhanced yields by growing a genetically pure hybrid crop. One of the problems faced by oil palm breeders is the difficulty in identifying true hybrids from the crossed progenies before planting. The traditional method of hybrid identification based on morphological characters is influenced by environmental factors and frequently lacks the resolving power to identify hybrids at the juvenile stage. Therefore, oil palm plants are to be grown to maturity (~ 3 to 4 years) to confirm hybridity.

A reliable method for identification of hybrid oil palm at the early stage of the plants is thus essential. Molecular markers used to detect DNA polymorphism are the most direct answer to the problem. DNA marker is a new approach based on DNA polymorphism among tested genotypes, and thus applicable to biological research. It offers many advantages over other categories of markers such as morphological, cytological or biochemical markers. For example, DNA marker can cover the whole genome and, therefore, is much larger in quantity. There is more polymorphism in DNA markers, which are able to reveal the variation and allelism. Many DNA markers are co-dominant and can differentiate between the homozygous and heterozygous genotypes. Furthermore, DNA markers are 'neutral', and they have no effect on phenotype, no epistatic effect, and are not influenced by environmental conditions and developmental

stages of plant tissues. Therefore, DNA marker is efficient, less effect of environment, and experimentally reproducible well. It has been applied widely in the identification, registration of plant variety, and in monitoring of the seed purity and the authenticity with high accuracy, high reliability and reasonable cost (Xin *et al.*, 2005). At present, the main DNA markers assisted selection in plant are restriction fragment length polymorphic (RFLP), random amplified polymorphism DNA (RAPD) and simple sequence repeat (SSR).

Currently, the random amplified polymorphic DNA (RAPD) technique based on the polymerase chain reaction (PCR) has been one of the most commonly used molecular techniques to develop DNA markers. RAPD markers are amplification products of anonymous DNA sequences using single, short and arbitrary oligonucleotide primers, and thus do not require prior knowledge of a DNA sequence. Low expense, efficiency in developing large number of DNA markers in a short time and requirement for less sophisticated equipment has made the RAPD technique valuable although reproducibility of the RAPD profile is still the centre of debate. RAPD marker offer powerful tools for the characterization of genetic variability, genotype identification, genetic analyses, selection and breeding purposes (Tingey and Tufo, 1993). In many species, RAPD has proven useful for revealing polymorphism among genotypes (Williams *et al.*, 1990). In *Elaeis*, RAPD markers have been employed for the analysis of genetic variation among African germplasm accessions (Shah *et al.*, 1994). This result has revealed that high levels of genetic variation in these accessions and 9 out of 20 primers were able to generate polymorphic product ranging in length from 0.2 kb to 2.3 kb. Whereas RAPD marker have been unsuccessfully used for the detection of somaclonal variants among regenerant populations (Rival *et al.*, 1998). Moretzhn *et al.* (2000) found that two RAPD markers (R11–1282 and T19–1046 primers) were identified to be linked on both sides of the sh^{+} locus on linkage. Moretzsohn *et al.* (2002) reported the genetic diversity of Brazilian oil palm (*E. oleifera*) germplasm collected in the Amazon forest. This result has revealed that 14 primers out of 96 primers were shown to be specific to oil palm, while twelve were specific to Brazilian accessions. It has

moderate level of genetic diversity as compared to oil palm accessions. Kanop (2005) found that only one primer (primer UBC731) could be identified the cultivars and clone of oil palm by RAPD analysis. Junmag *et al.* (2004) studied the genetic variability and phylogenetic relationships in oil palm by RAPD analysis. This result has revealed that seven primers (OPB08, OPR11, OPT06, OPT19, OPAB01, OPAB09 and OPAB14) out of 160 primers were identified genetic variation in 151 individuals representing 52 dura, 60 tenera and 39 pisifera. A similarity index showed that relatively high levels of 0.6. So far, there has no report on verification of hybridity from cross combination in oil palm.

Among DNA markers, SSR has much more polymorphism than others, and is co-dominant and large in quantity. Therefore, SSR has become an ideal molecular marker in identification of plant variety. Zhan *et al*. (2002) tested six hybrid combinations and their parents of rice by using 178 SSR primers, of which 52 showed stable polymorphic patterns in one or more hybrid combinations, and two SSRs could be used to detect the purity of V46 and Jinyou 207 F1 seeds. Development of oil palm SSR markers has been used for measuring genetic diversity, variety identification, pedigree analysis and genome mapping and for quality trait loci (QTL) detection for marking assisted selection (MAS) (Jones, 1989; Brown, 1993; Jack and Mayes, 1993; Mayes *et al*., 1996a,b). Cheah *et al*. (1995) demonstrated that simple repetitive DNA was present in abundance in the oil palm. They screened oil palm genomic DNA for di, tri, tetra and penta nucleotide repeats and found them to be widely distributed in the oil palm genome. Initial attempts to construct SSR enriched genomic libraries were not successful, as less than 1% of the clones appeared to contain SSR motifs (Cheah and Ooi, 1999). Later, other researchers, Billotte *et al*. (1999) described an efficient technique for building microsatellite enriched libraries. This technique allowed the construction of several oil palm $(GA)_n$, $(GT)_n$ or $(CCG)_n$ enriched-libraries from total genomic as well as chloroplast DNA (cpDNA). About 200 functional SSR primer pairs have already been developed from microsatellite clones and have been sequenced. Billotte *et al*. (2001) characterized 21 SSR loci together with primer sequences; estimation of allele size range were made, as well as expected heterozygosity in *E.*

guineensis and in the closely related species *E. oleifera,* where an optimal utility of the SSR markers was observed. High levels of allelic variability indicate that *E. guineensis* SSRs are putative markers for all palm taxa. In addition, phylogenetic information based on SSR flanking region sequences makes *E. guineensis* SSR markers a potentially useful molecular resource for study of palm taxa phylogeny. SSR will be powerful for genetic studies of the *Elaeis* genus for variety identification and intraspecific or interspecific genetic mapping. Bakoume (2006) used the oil palm genomic SSR marker developed for oil palm by Billotte *et al.* (2001) to extensively screen the Cameroon collection. These studies have demonstrated the usefulness of molecular markers to assess the genetic variability for cultivar protection and establishment of core collections for conservation of the oil palm gene pool. Recently Singh *et al.* (2007) developed SSR markers from an oil palm expressed sequence tags (ESTs) database. The search revealed that the oil palm ESTs contained mono-, di-, tri- and tetra-nucleotide motifs. All the SSRs were found in ESTs and they are expected to become useful tools for the oil palm ecological, genetic and evolutionary studies. Dimeric repeats, especially AG/CT, were the most abundant. The EST-SSR was able to estimate the genetic diversity as well as determine the genetic relationship between the different germplasm collections. However, there are no researches or reports with hybridity testing of oil palm.

In the present paper, we describe the method for verifying F1 hybrid of various crosses of oil palm using half zygotic mature embryos (HZMEs) by RAPD and SSR markers.

Materials and Methods

Plant material

Mature zygotic embryos (MZE) of 'Tenera' F1 hybrid, derived from six crosses; cross #58, cross #77, cross #118, cross #119, cross #130 and cross #137 at 180 days after pollination (DAP), were kindly provided by Assoc. Prof. Dr. Theera Eksomtramage (Agricultural Research Station, Klong Hoi Khong, Songkhla, Thailand)

and were excised by the following protocol. The mesocarps were completely removed from the fruits. The seeds were gently cracked and the embryos, surrounded by kernel, were carefully trimmed to form a small cube of size 5x5x8 mm and used as explants for culture. The cubes were surface sterilized by 70% alcohol for 1 minute and 20% Clorox (containing 0.5 ml of Tween-20 emulsifier per 100 ml solution) for 20 minutes, followed by successive washing with sterile distilled water 3 times in a laminar flow station. The embryos were then aseptically removed from the kernel and cultured on culture medium.

DNA isolation and quantification

Total genomic DNA was extracted from freeze-dried adult leaf tissue of the parents using a modified Hexadecyltrimethyammonium bomide (CTAB) protocol (Doyle and Doyle, 1990). In case of HMZEs, they were derived from MZEs and were cultured on MS medium supplemented with either 40 mg/l NAA or 2,4-D or dicamba at concentrations of 2.5 and 5.0 mg/l for 4 weeks. Elongated MZEs with fully developed haustorium were cut in half (cross section). The first half of MZEs (HMZEs), consisting mainly of haustorium, were collected (approximately 15-20 mg) and brought to DNA isolation according to the technique describe by Te-chato (2000). DNA from leaf tissue of the parents and from HMZEs of hybrids oil palm were quantified comparing the fluorescence intensities of the ethidium bromide treated samples to those of a λ DNA standard dilution series in 0.75% agarose gel electrophoresis under UV light with gel documentation.

Hybrid verification via RAPD analysis

RAPD analysis of genomic DNA was carried out using 7 decamer random oligonucleotide primers (OPB08, OPR11, OPT06, OPT19, OPAB01, OPAB09 and OPAB14) obtained from Operon Tech. (California, USA). The RAPD analysis was performed according to the methodology of Junmark (2004). Each amplification mixture

of 25 µl contained 2.5 mM $MgCl_2$, 10x *Taq* buffer, 100 µM of each dNTP, 0.3 mM of primer, 1.5 units of *Taq* polymerase and 20 ng of template DNA. PCR amplification were carried out on a thermocycler using the following profile was started from 1 cycle of 95°C for 1 min, 39 cycles of 95°C for 1 min 37°C for 1 min 72°C for 2 min, followed by 1 cycle of 95°C for 1 min 37°C for 1 min and finally 72°C for 10 min. PCR products were then electrophoresed in 1.5% (w/v) agarose gels in 0.5x TBE buffer at 100 V. The gels were stained with ethidium bromide for 15 min and stained with distilled water for 10 min and then viewed under ultraviolet light with gel documentation. Reproducibility of the amplification patterns was verified by using different DNA preparations from the parent.

Hybrid verification via SSR analysis

MZEs were cultured on MS supplemented with either 40 mg/l NAA or 2, 4-D or dicamba at the concentration of 2.5 and 5.0 mg/l for 4 weeks. Elongated MZEs with fully developed haustorium way cut in to half (cross section). First HMZE consisted of mainly haustorium were collected (at approximately 15-20 mg) and isolated DNA. SSR analysis of genomic DNA was carried out using 9 microsatellite loci amplified in oil palm. SSR analysis of genomic DNA was carried out using 9 primers (EgCIR008, EgCIR0243, EgCIR0337, EgCIR0409, EgCIR0446, EgCIR0465, EgCIR0781, EgCIR0905 and EgCIR1772) obtained from Operon Tech. (California, USA). The details of those primer sequences were shown in Table 9. Amplification of genomic DNA was done according to the protocol of Billotte *et al.* (2005). Each amplification mixture of 10 µl contained 2.5 mM $MgCl_2$, 10x *Taq* buffer, 100 µM of each dNTP, 0.3 mM of primer, 1.5 units of *Taq* polymerase and 20 ng of template DNA. PCR amplifications were carried out on a thermocycler (TC-XP-G, Japan) using the following program: denaturation at 95°C for 1 min; 35 cycles of 94°C for 30 s, 52°C for 60 s, and 72°C for 120 s; and a final elongation step at 72°C for 8 min. An equal volume of loading buffer (98% formamide, 0.025% bromophenol blue, 0.05% xylene cyanol) was added to the amplified products, following denaturation at 94°C for 5 min. The products were separated on 6% (w/v) denaturing

polyacrylamide gels. Silver staining was conducted according to the protocol provided by Bassam *et al.* (1991).

Results

DNA isolation and quantification

For leaf tissue of the parents using a modified CTAB protocol, up to 80 μg of DNA were routinely obtained from a minimal amount of 200 mg of fresh leaf oil palm of an average fragment size. This extraction method consistently produced a high yield of good-quality DNA (Figure 14). HMZEs DNA isolation of hybrids oil palm followed by Te-chato (2000) procedure gave DNA yields of 15–25 μg/20mg. HMZEs, as estimated by agarose gel electrophoresis (Figure 15). The DNA produced by both methods showed little degradation and were sufficient for PCR reactions in oil palm genomic analysis. Because only 10-20 ng of DNA is needed per reaction of PCR-based RAPD or SSR analysis, the DNA obtained from each sample is sufficient for more than 50 PCR reactions as well as for other PCR-based marker identification and verification of oil palm.

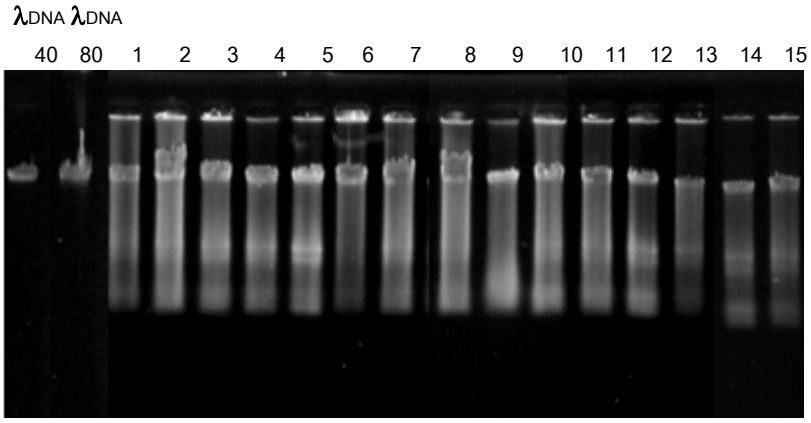


Figure 14 Demonstration of the quality of leaf parents DNA obtained by the modified CTAB method.

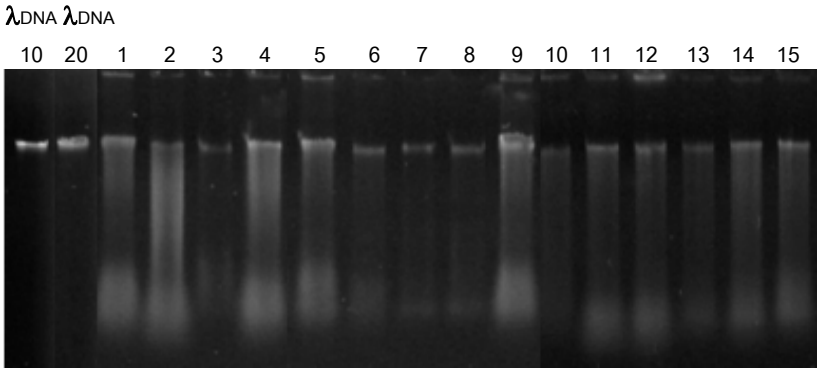


Figure 15 Demonstration of the quality of HMZEs DNA obtained by the Te-chato method.

Hybrid verification via RAPD analysis

All primers could be amplified and provide polymorphic patterns among parents. The number of bands for each primer varied from 8 to 17 with an average of 13.86 fragments per primer. The size of the amplified products ranged from 100 to 1517 bps. A total of 97 RAPD fragments were scored from the seven random primers (Table 6). The results revealed that there was only one primer, OPT06 provided clearly DNA pattern (Figure 16). OPT06 primer had the greatest capacity for discriminating polymorphic fragments in half-embryo cultured and gave the highest percentage of polymorphic at 87.50, followed by OPT19 (86.67) and OPB08 (65.54), respectively.

Cross #77 and cross #119 were a good model for verification of hybrid with OPT06 primer (Figure 17). Among hybrid tested, three main types of DNA pattern were observed: non-amplified band, not-clear band and specific-hybrid band (Table 7). Cross #77 gave the highest number of non-amplified band at 61, followed by specific-hybrid band and not-clear band which provided number of DNA pattern at 24 and 15, respectively. The result also revealed that cross #77 gave the percentage of hybridity at 24.00. For cross #119 gave the highest number of non-amplified band at 110, followed by specific-hybrid band and not-clear band which provided number of DNA pattern at 40 and 18, respectively. The result also revealed that cross #119 gave the percentage of hybridity at 23.81.

Specific fragment (650 bps) from primer OPT06 was formed in male parent (72P) and this fragment can be used to distinguish hybrids from their parental plant. Primer OPT06 was proved to be hybrid because they present a 650 bps fragment, a specific band from 172P (Figure 18). Similarity results were obtained among progeny of cross #119, a 400 bps fragment from primer OPT06 could be used to verify from cross 865(D) × 206 (P) (Figure 19).

Table 6 Primers used in RAPD analysis of identified genetic in the parents plant of *Elaeis quineensis* Jacq. and number of scoreable bands for each primer.

Primer	Sequence fragments	Amplified fragments	Monomorphic fragments	Polymorphic fragments	Polymorphic (%)	Band mol. Weight (bps)
OPB08	GTCCACACGG	13	5	8	65.54	150-1480
OPT06	CAAGGGCAGA	8	1	7	87.50	290-1100
OPT19	GTCCGTATGG	15	2	13	86.67	100-2100
OPR11	GTAGCCGTCT	16	7	9	56.25	200-1517
OPAB01	CCGTCGGTAG	13	9	4	30.77	300-1500
OPAB09	GGGCGACTAC	17	6	11	64.71	210-1500
OPAB14	AAGTGCGACC	12	9	3	25.00	210-1517
Total band scored		97	40	57	58.76	

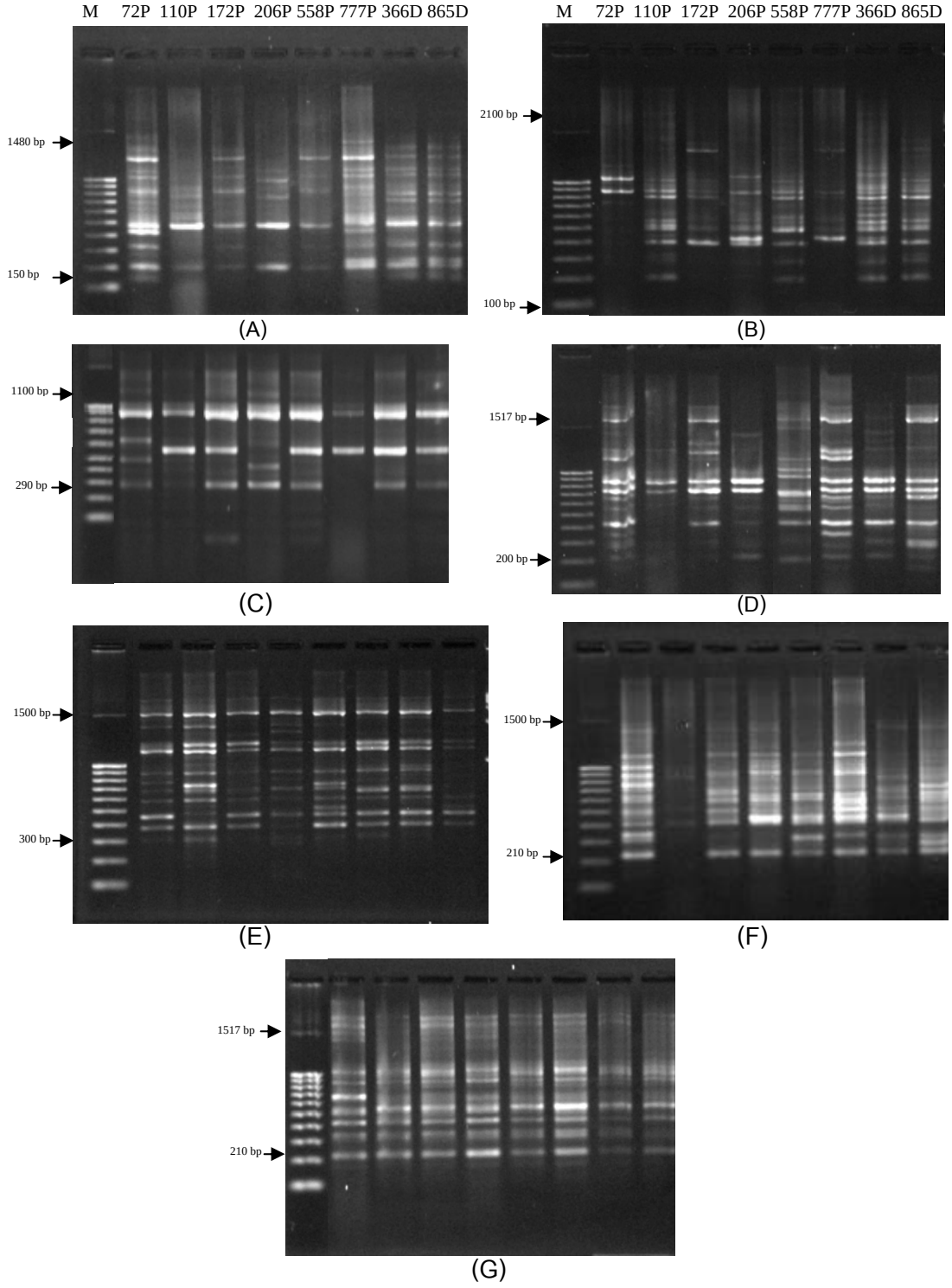


Figure 16 The amplification products were compared on the basis of molecular size. Lane 1, 2, 3, 4, 5 and 6 were fragments from pisifera plants. Lane 7 and 8 were fragments from dura plants, obtained with primers OPB08 (A), OPT19 (B), OPT06 (C), OPR11 (D), OPAB01 (E), OPAB09 (F) and OPAB14 (G).

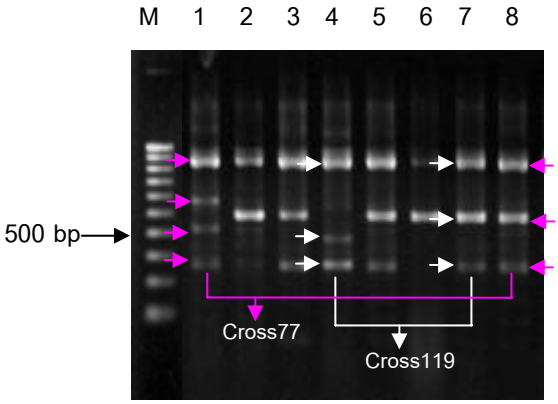


Figure 17 Randomly amplified polymorphic DNA patterns in parents. The amplification products were compared on the basis of molecular size. Lane 1, 2, 3, 4, 5 and 6 were fragments from pisifera plants. Lane 7 and 8 were fragments from dura plants, obtained with primers OPT06.

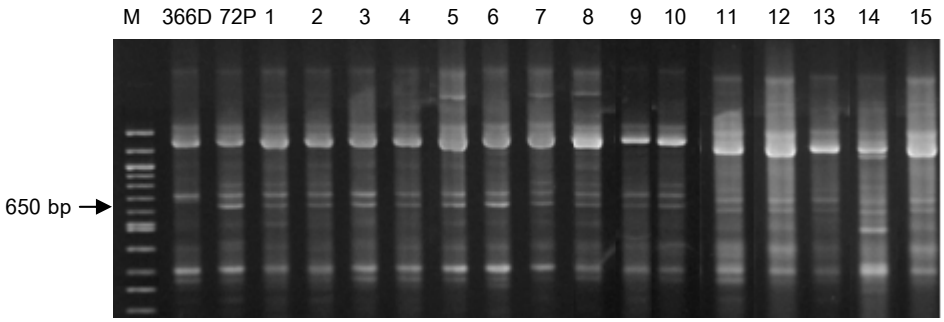


Figure 18 RAPD patterns in hybrids and parents of cross #77. The amplification products were compared on the basis of molecular size. Lane P and D were fragments from parents. Lane 1-15 were fragments from hybrids obtained with primers OPT06

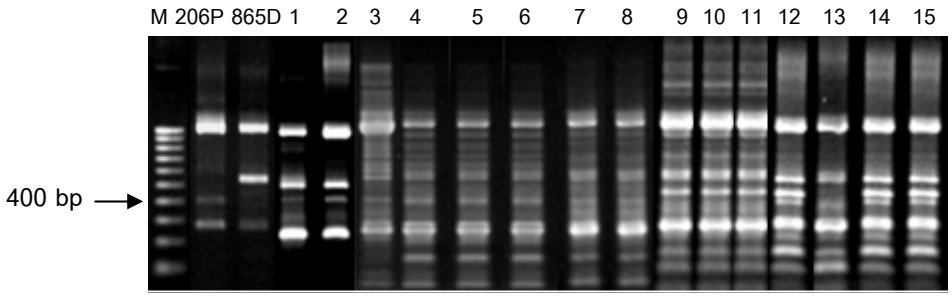


Figure 19 RAPD patterns in hybrids and parents of cross #119. The amplification products were compared on the basis of molecular size. Lane P and D were fragments from parents. Lane 1-15 were fragments from hybrids obtained with primers OPT06.

Hybrid verification via SSR analysis

A set of 9 SSR primers (EgCIR008, EgCIR0243, EgCIR0337, EgCIR0409, EgCIR0446, EgCIR0465, EgCIR0781, EgCIR0905 and EgCIR1772) was selected to evaluate polymorphism levels among eight parents of hybrid oil palm. All primers could be amplified and provide polymorphic patterns of DNA from the parents. The number of alleles per locus varied from 1 for EgCIR0337 to 8 for EgCIR0008 with a mean of 4.89±2.09 alleles per locus. The size of the amplified DNA ranged from 100 to 250 bps. The majority of polymorphic SSR loci generated five alleles at 33.33% and other each alleles (one, tree, four, six, seven and eight alleles) gave polymorphic loci at 11.11% (Table 7). The results revealed that two primers, EgCIR008 and EgCIR1772 primers provided clearly DNA pattern and produced codominant marker (Figure 20 and 21).

Two further SSR markers proved to be suitable for six different hybrid combinations. For EgCIR008 primer, this primer with the highest information capacity could be applied for more than one cross combination: cross #77, cross #118, cross #119 and cross #137 distinguished between the parents of four different hybrid combinations (Figure 22a). While EgCIR1772 primer, cross #58 and cross #130 were able to identify two different hybrid combinations (Figure 22b).

To test the reliability of the microsatellite-based hybrid identification technology, 100 F1 individuals of cross #77 was screened for heterozygosity with EgCIR008 primer. Cross #77 showed specific fragment and could be used to distinguish hybrid between dura and pisifera. All hybrids were heterozygous with primer mEgCIR008 showing both of male parent band (115 and 120 bps) and female parent band (105 bps) (Figure 23A). In the cross #118 with primer EgCIR008, the hybrid produced both fragments from dura and pisifera that confirmed hybridity (Figure 23B). One hundred samples were heterozygous with primer mEgCIR008 showing both of male parent band at 115 bps and female parent band at 105 bps. With the identified primer 100 individuals in cross #119 showed heterozygous with primer EgCIR008 showing the presence of both of male parent band (115 bps) and female parent band (105 bps) (Figure 23C). The result also revealed that cross #119 gave the percentage of hybridity at 100. For cross #137 with primer EgCIR008 produced specific fragment and could be used to distinguish hybrid between dura and pisifera. All hybrids were heterozygous with primer EgCIR008 showing the presence of both of male parent band (115 and 120 bps) and female parent band (105 bps) (Figure 23D).

In the cross #58 with primer EgCIR1772, the hybrid produced both fragments from dura and pisifera that confirmed hybridity. One hundred samples were heterozygous with primer mEgCIR008 showing both of male parent band at 110 and 115 bps and female parent band at 105 and 108 bps (Figure 24A). The result revealed that cross #130 showed specific fragment and could be used to distinguish hybrid between dura and pisifera with EgCIR1772 primer. The hybrids were heterozygous with primer mEgCIR1772 showing both of male parent band (110 and 115 bps) and female parent band (105 and 108 bps). However, only one hybrid was homozygous with primer EgCIR1772 showing the presence of male parent band only (Figure 24B).

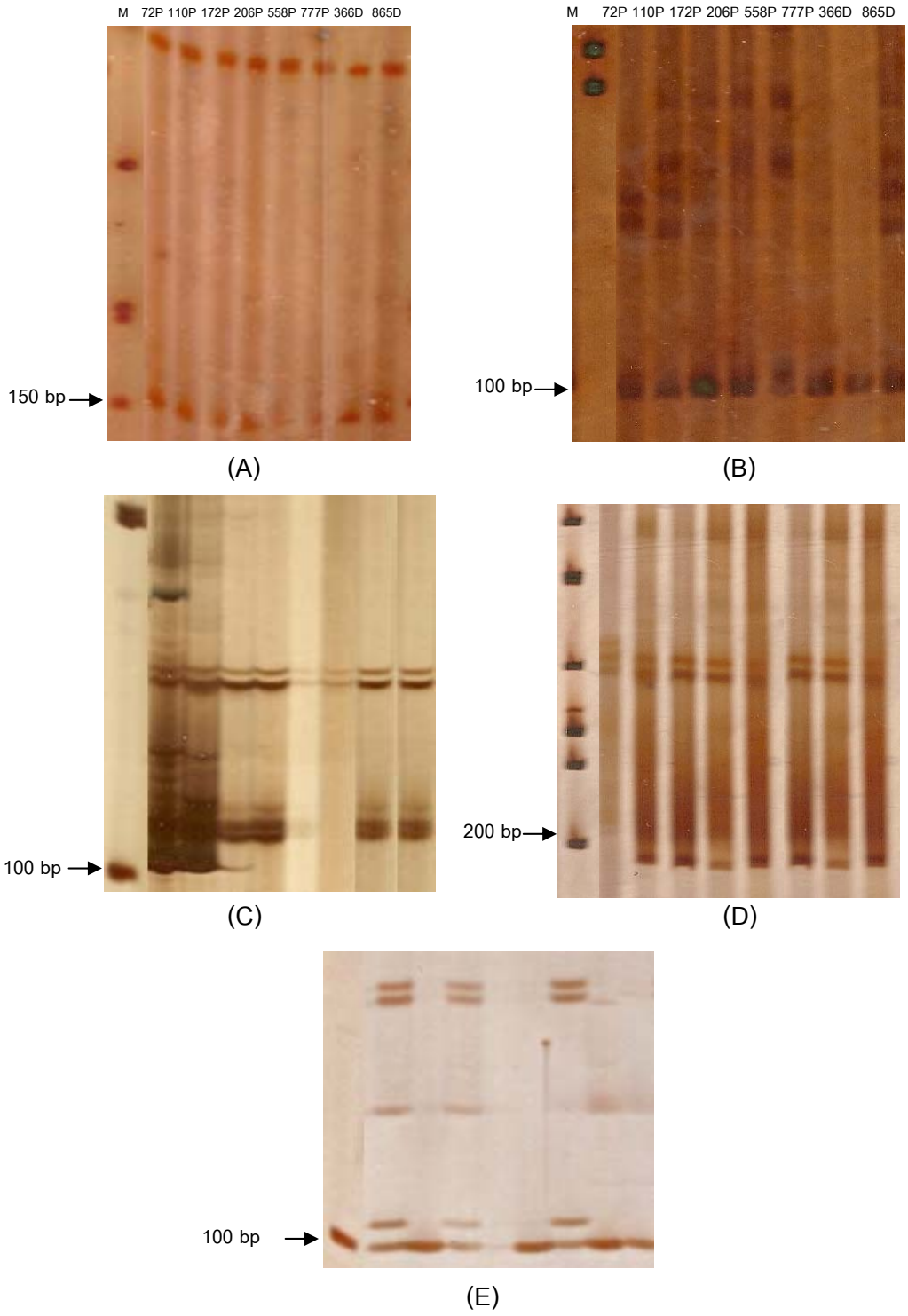


Figure 20 Amplification patterns of SSR locus of parents. The amplification products were compared on the basis of molecular size. Lane 1, 2, 3, 4, 5 and 6 were fragments from pisifera plants. Lane 7 and 8 were fragments from dura plants, obtained with primers EgCIR0337 (A), EgCIR0409 (B), EgCIR0781 (C), EgCIR0905 (D) and EgCIR0465.

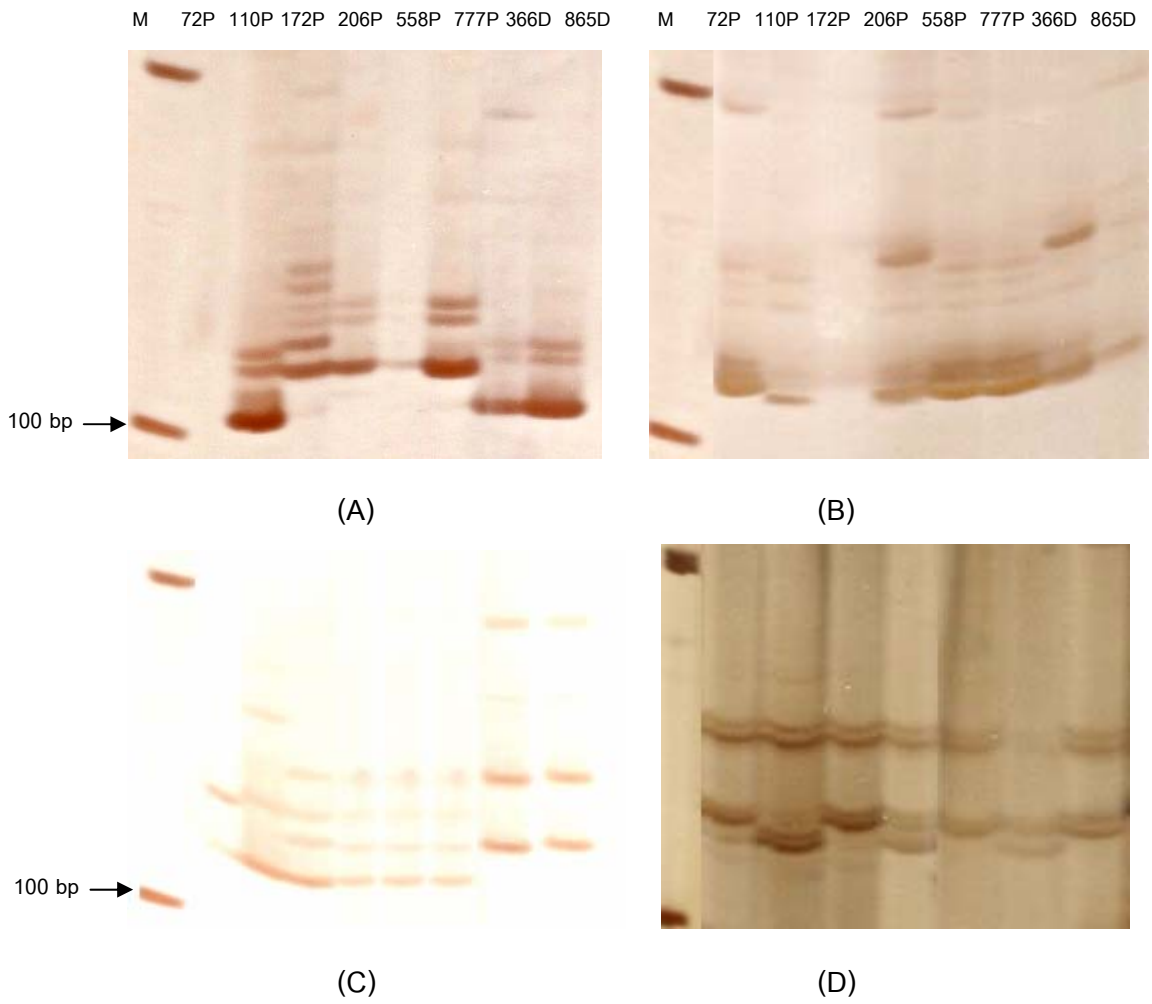
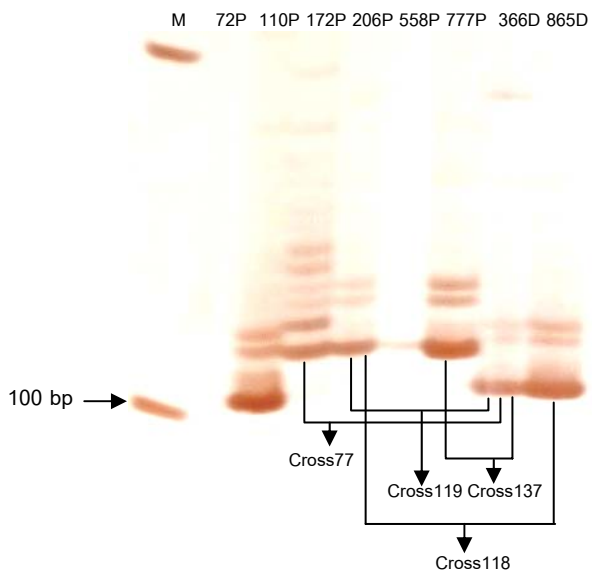
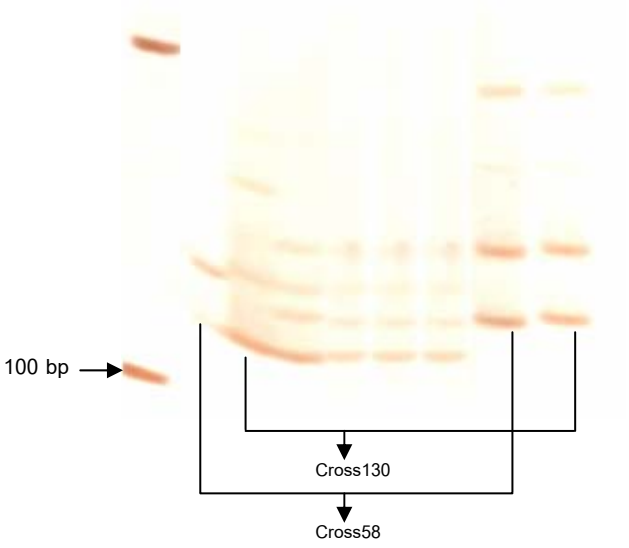


Figure 21 Amplification patterns of SSR locus of parents. The amplification products were compared on the basis of molecular size. Lane 1, 2, 3, 4, 5 and 6 were fragments from pisifera plants. Lane 7 and 8 were fragments from dura plants, obtained with primers EgCIR0008 (A), EgCIR0446 (B), EgCIR1772 (C), and EgCIR0230 (D).



(A)



(B)

Figure 22 Amplification patterns of SSR locus of parents. The amplification products were compared on the basis of molecular size. Lane 1, 2, 3, 4, 5 and 6 were fragments from pisifera plants. Lane 7 and 8 were fragments from dura plants, obtained with primers EgCIR0008 (A) and EgCIR1772 (B).

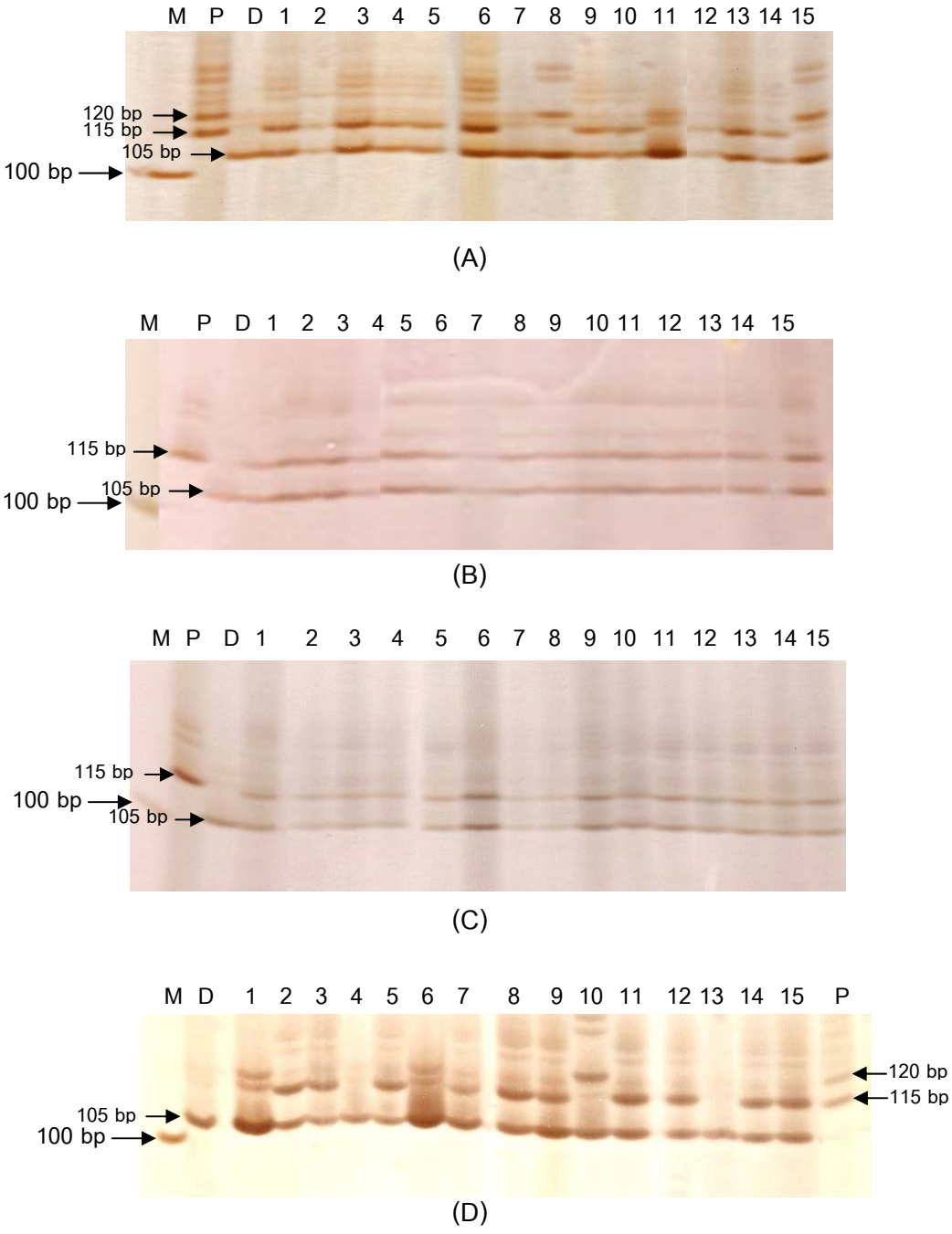


Figure 23 SSR patterns in hybrids and parents of cross #77 (A), cross #118 (B), cross #119 (C) and cross #137 (D) obtained with primer EgCIR0008. The amplification products were compared on the basis of molecular size. Lane P and D were fragments from parents. Lane 1-15 were fragment from hybrids. The arrows indicate MPS and FPS bands.

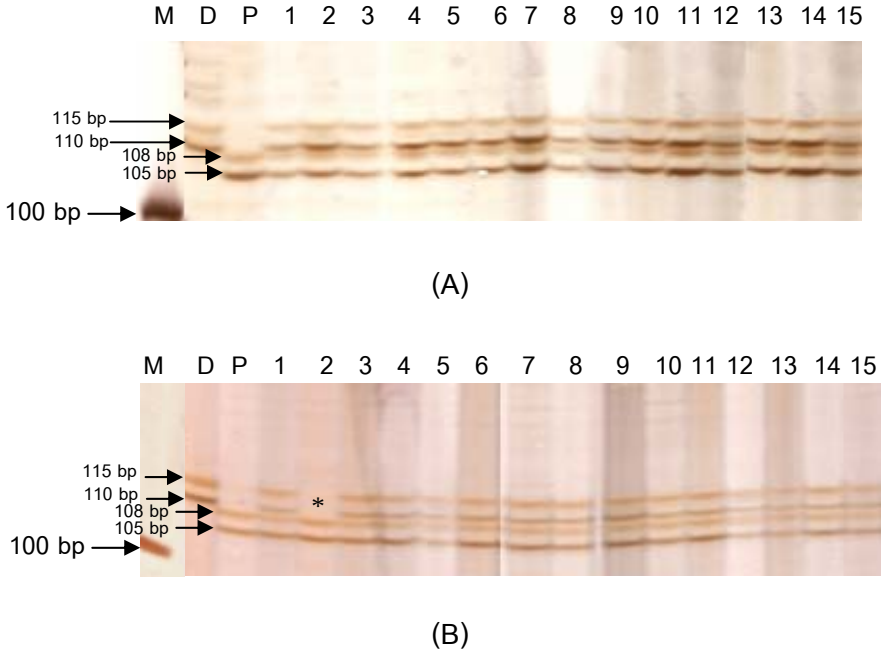


Figure 24 SSR patterns in hybrids and parents of cross #58 (A) and cross #130 (B) obtained with primer EgCIR1772. The amplification products were compared on the basis of molecular size. Lane P and D were fragments from parents. Lane 1-15 were fragment from hybrids. The arrows indicate MPS and FMS bands. The symbol (*) denoted this hybrid that did not hybrid.

Discussion

DNA isolation and quantification

The use of RAPD as a routine method for verification of hybrids oil palm for the commercial scale and for the distributation to farmers is DNA quality. For this reason, we confirmed a modified DNA extraction of CTAB method from leaf tissue parents and a relatively faster DNA isolation of Te-chato (2000) method from HMZEs of hybrids oil palm. Both oil palm tissue types and both DNA extraction methods tested were provided highly reproducible results for the selected RAPD markers. For leaf tissue, DNA isolation by modified CTAB method gave intensively stained bands and should be chosen suitable for RAPD marker. This method used CTAB (Hexadecyl trimethyl-ammonium bromide, CTAB) that can be used to liberate and complex with total cellular nucleic acids from a wide array of plant genera and tissue types, especially a very fiberous tissue (Rahimah *et al*., 2006). It was indicated that more intensely stained bands should be chosen as RAPD markers while Shah *et al.* (1994) used the method of Dellaporta *et al.* (1983) for isolated DNA from leaf tissue of oil palm. However, Rival *et al*. (1998), Moretzsohn *et al.* (2000), Moretzsohn *et al.* (2004) and Junmag *et al.* (2004) used CTAB method for isolated DNA from leaf tissue of oil palm. It also provided the highest a mount of DNA from oil palm leaf tissue. In this study, a large amount of DNA from each hybrids of oil palm could be extracted from HMZEs (very small tissue, ~ 10-20 mg) by the use of Te-chato (2000). This method is modified from Dellaporta *et al.* (1983) and also performed similarity with Te-chato *et al.* (2005) in *Lansium* spp. and Promchan (2008) in oil palm. DNA is extracted with dialyzed against SDS, EDTA and a buffered Tris-HCl solution. The proteins and other cellular were removed by ammonium acetate. This procedure does not use liquid nitrogen and toxic chemical such as chloroform. It is simple, efficient and produced DNA suitable for SSR and RAPD analysis. It is the most suitable for a small amount plant tissue. Based on our experiment, we concluded that both DNA isolation methods can be used as a suitable method for extracted DNA in oil palm.

Hybrid verification via RAPD analysis

A high reproducibility of RAPD patterns is a prime requirement for the use of the RAPD method in hybrid verification. Care must be taken during all the operations involved in the RAPD reaction. Precise quantification of DNA after purification is therefore a crucial step. In the present study, RAPD marker was employed to evaluate 6 crosses of hybrids oil palm. The result of our experiment indicated that RAPD marker can be used for verification of hybrid. Out of 7 primers, only one primer, OPT06, provided clearly DNA pattern. Cross #77 obtained from the parent 366 (Dura) × 172 (Pisifera) and cross #119 obtained from the parent 865 (Dura) × 206 (Pisifera) were a good model for verification of hybrid with OPT06 primer. These results were similar to those reported by Carvalho *et al.* (2004) in chestnut. They indicated that RAPD marker was successfully use for detecting the material propagated *in vitro* and the donor plants of chestnut. However, some bands were amplified from the F1 hybrid but not from parents. The reason could be due to the complex heredity background of each plant heterosis (Chowdhury and Vasil, 1993; Cordeiro *et al*., 2000; Chen *et al*., 2004). Additionally, this residual heterozygosity could account for the occasional occurrence that some true hybrids exhibited the absence of the female or male specific-parent markers with RAPD primer. Liu *et al.* (2007) reported that primer NAURP 403 and 1123 produced female parent-specific marker only while primer NAURP409 can produce both of female and male parent-specific markers simultaneously in the hybrid of tomato. Similarly, residual amounts of heterozygosity can also result in a selfed parental plant generating the female and male parent-specific markers in some marker loci. RAPD marker sensitive to the conditions under which the analysis is conducted may create problems with reproducing experiments under conditions of another laboratory (Devos and Gale, 1992). Due to these potential problems with RAPD, PCR reactions of the analyses presented in this study were performed in two replications for screening parent with 7 primers. However, our results from this present study showed that RAPD marker was an effective tool for verification and identification hybridity of oil palm.

Hybrid verification via SSR analysis

SSR markers with the high information content can be used for identification of several genotype and hybrid combinations. SSR markers have been successfully applied for cultivar and hybrid identification in a number of plant species (Becher *et al*., 2000; Esselink *et al*., 2003; Gémes Juhász *et al*., 2002; Tessier *et al*., 1999; Vosman *et al*., 2001; Yashitola *et al*., 2002). Before stepping to that point, hybridity of MZE should be verified at an early stage subsequent to mass propagation. In this present study, we were successfully developed the verification technique of hybrids oil palm by SSR analysis using HMZEs-cultured. Nine primers are informative enough to distinguish all the parental lines and hybrid consideration. Among those primers tested, EgCIR008 generated both male and female parent band in their hybrids of cross #77, cross #118, cross#119 and cross #137. Whereas the hybrid of cross #58 and cross #130 can be verified by primer EgCIR1772, Similar results were also reported in *Capsicum* (Mongkolporn *et al.,* 2004), cotton (Dongre *et al.,* 2005), cucumber (Watcharawongpaiboon and Chunwongse, 2008) and rice (Sundarm *et al.,* 2008; Garg *et al*., 2006). This technique is suggested to be a potential way for identification hybrid in a large numbers of heterozygous plant species. The breeding program, selection phenotypic traits is proved to be primarily needed. However, for perennials or fruit trees, it is time consumed for the use of interested trait for selection. Therefore, molecular markers can be assisted to select the desirable traits in a short period of time. The important gene coding regions of the genome and near locations get fixed quickly for the respective alleles; otherwise, the markers get fixed at random, hence showing plant to plant variation within parental lines (Garg *et al*., 2006). The result of the present study showed that individuals of the cross #130 could be true hybrids, except one sample that generated only female parent band with the primer EgCIR1772. These results were similar to those reported by Lui *et al.* (2007) in tomato. They indicated that SSR marker was successfully use for detecting the material propagated *in vitro* and the donor plants of tomato. However, eight of the 210 F1 plants in 'Hezuo 903' was produced female parent band only with primer NAUSSRTO2 the same as thirteen of 210 F1 plants in

‘Sufen No. 8’ was generated male parent band only with primer NAURP409. Similarly, residual amounts of heterozygosity can also result in a self parental plant generating male parent band and female parent band in some marker loci. Therefore, it is suggested that only one sample of cross #130 did not be hybrids but may derived from female-parental plant. Considering the residual heterozygosity in parental lines and all hybrids of oil palm, it can be concluded that codominant SSR marker was a great tool for hybridity verification. It was quick, costly, effective and using a combination of multiple markers would be a little laborious.

CHAPTER 4

Screening and detection of hybrids oil palm by DNA marker

Experiment II

Assessment of somaclonal variation by RAPD and SSR analysis

CHAPTER 4

EXPERIMENT II

Introduction

Nowadays plant micropropagation is used for plant breeding, and clonal propagation oil palm through tissue culture is common (Te-chato and Muangkaewngam, 1992; Rajesh *et al*., 2003). There have been previous reports of oil palm micropropagation through somatic embryogenesis (Te-chato, 1998b; Te-chato and Hilae, 2007; Chemalee and Te-chato, 2008). Somatic embryogenesis has been regarded as the *in vitro* system of choice with the potential for cell and genetic engineering of oil palm and characterized by the formation of an external bipolar structure from plant tissues without connection to the plant vascular system; moreover, this structure bears root and shoot poles. However, the most of somatic embryo induction was success when induced on high concentration of 2,4-D containing medium (Ward and Jordan, 2001; Teixeira *et al*., 1993). The use of high concentration of 2,4-D has been implicated as a cause of somaclonal variation in different species of plant, including oil palm (Teixeira *et al*., 1994; 1995). Moreover, somaclonal variation generated during *in vitro* culture, it cannot be certified that the genetic organization of tissue culture derived material is identical to the explant from which it originates and that it is inherently homogeneous (Corniquel and Mercier, 1994). The assessment of the genetic stability of *in vitro* derived clones is an essential step in the application of biotechnology for micropropagation of true to type clones (Diaz *et al*., 2003). Identification of off-types and genetically not identical to the mother plant at an early stage of development is considered to be very useful for quality control in plant tissue culture. So the use of molecular markers is becoming widespread for the identification of somaclonal variants and the assessment of *in vitro* regeneration protocols (Taylor *et al*., 1995). RAPD (Randomly Amplified Polymorphic DNA) is a valuable tool for identifying genetic variation because it is inexpensive, quick and simple (Williams *et al*., 1990). RAPD

marker offer powerful tools for the characterization of genetic variability, genotype identification, genetic analyses and selection and breeding purposes (Tingey and Tufo, 1993). It permits identification of DNA polymorphisms and can be used to amplify particular fragments of genomic DNA (Bielawski *et al*., 1996). DNA profiles have been electrophoresed to analyze the genetic relationships of plant species (Ayana *et al*., 2000). RAPD analysis is based on the presence or absence of polymorphisms in individuals or groups of individuals (Tingey and Tufo, 1993). In *Elaeis*, RAPD markers have been employed for the analysis of genetic variation among African germplasm accessions (Shah *et al*., 1994). This result has revealed that high levels of genetic variation in these accessions and nine out of 20 primers were able to generate polymorphic product ranging in length from 0.2 kb to 2.3 kb. Whereas RAPD marker have been unsuccessfully used for the detection of somaclonal variants among regenerant populations (Rival *et al*., 1998). SSR markers have also been shown to have transferability across different species and plants, a fact that increases their value in plant genetic studies (Peakall *et al*., 1998). As codominant and locus specific markers, SSRs have been widely used as tools in genotype identification (Billotte *et al*., 2001), genetic mapping (Billotte *et al*., 2005) and population genetic (Singh *et al*., 2007) studies in oil palm. The above success RAPD marker proved genetic change and selected, it is of great important in propagation of oil palm in commercial scale. From chapter 4.1, DNA pattern among cross #77 and cross #119 were found specific fragment at 650 and 400 bps, respectively. However, the latter cross could not promote completed plantlets. Therefore, only cross #77 was a good model for detection true to type. Thus, it is utilized for detection and confirmation of hybridity in this experiment. These studies have confirmed the importance of SSR as a source of markers for oil palm genetics. However, there are no researches or reports detection and confirmation of micropropagated plantlets of oil palm. The aim of this chapter was to detect uniformity of somatic embryos at globular stage, regenerated plantlet lines and their mother plants of cross #77 by RAPD marker and detected uniformity of six cross combinations of oil palm by SSR marker.

Materials and Methods

Plant materials

HMZEs of 'Tenera' hybrid, derived from six genotypes, cross #58, cross #77, cross #118, cross #119, cross #130 and cross #137, at 180 days after pollination (DAP) were kindly provided by Assoc. Prof. Dr. Theera Eksomtramage (Agricultural Research Station, Klong Hoi Khong, Songkhla, Thailand) and excised by the following protocol. The mesocarps were completely removed from the fruits. The seeds were gently cracked and the embryos, surrounded by kernel, carefully trimmed to form a small cube of size 5x5x8 mm and used as explants for culture. The cubes were surface sterilized by 70% alcohol for 1 minute and 20% Clorox (containing 0.5 ml of Tween-20 emulsifier per 100 ml solution) for 20 minutes, followed by successive washing with sterile distilled water for 3 times in a laminar flow station. The embryos were then aseptically removed from the kernel and cultured on culture medium.

DNA isolation and quantification

Freeze-dried adult leaf tissue of the parent and young leaf obtained from regenerated plantlets were isolated DNA following by the method described in chapter 4.1. For DNA extraction of somatic embryos at globular stage, they were conducted according to the protocol provided by Te-chato (2000).

Assessment of somaclonal variation by RAPD analysis

DNA samples of somatic embryos at globular stage from cross #77, regenerated plantlets lines and their mother plant were amplified with OPT06 primer. The RAPD analysis was performed according to the methodology of Jungmark (2004). Each amplification mixture of 25 µl contained 2.5 mM $MgCl_2$, 10x *Taq* buffer, 100 µM of each dNTP, 0.3 mM of primer, 1.5 units of *Taq* polymerase and 20 ng of template DNA.

The thermal profile for RAPD-PCR was started from 1 cycle of 95°C for 1 min, 39 cycles of 95°C for 1 min 37°C for 1 min 72°C for 2 min, followed by 1 cycle of 95°C for 1 min 37°C for 1 min and finally 72°C for 10 min. PCR products were then electrophoresed in 1.5% (w/v) agarose gels in 0.5X TBE buffer at 100 V. The gels were stained with ethidium bromide for 15 min and viewed under ultraviolet light with gel documentation. The amplification products were compared with the parent plant pattern in order to detect any change produced after the culture period.

Assessment of somaclonal variation by SSR analysis

DNA samples of somatic embryos at globular stage from all cross combinations, regenerated plantlets lines and their mother plant were amplified with EgCIR0008 and EgCIR1772 primers. The SSR analysis was done according to the protocol of Billotte *et al.* (2005). Each amplification mixture of 10 µl contained 2.5 mM $MgCl_2$, 10x *Taq* buffer, 100 µM of each dNTP, 0.3 mM of primer, 1.5 units of *Taq* polymerase and 20 ng of template DNA. PCR amplifications were carried out on a thermocycler (TC-XP-G, Japan) using the following program: denaturation at 95°C for 1 min; 35 cycles of 94°C for 30 s, 52°C for 60 s, and 72°C for 120 s; and a final elongation step at 72°C for 8 min. An equal volume of loading buffer (98% formamide, 0.025% bromophenol blue, 0.05% xylene cyanol) was added to the amplified products, following denaturation at 94°C for 5 min. The products were separated on 6% (w/v) denaturing polyacrylamide gels. Silver staining was conducted according to the protocol provided by Bassam *et al.* (1991).

Results

Assessment of somaclonal variation by RAPD analysis

Somatic embryos at globular stage, regenerated plantlet lines and their mother plants from the parent 366 (Dura) × 172 (Pisifera) were detected their uniformity using RAPD technique. Primers used in this technique were shown to amplify the products of DNA in Figure 25 and 26. Among 15 of somatic embryos and regenerated plantlets there was no variation detect in DNA profile amplified by this primer. Thus, we concluded that there is no somaclonal variation occurred in our propagation system by RAPD marker.

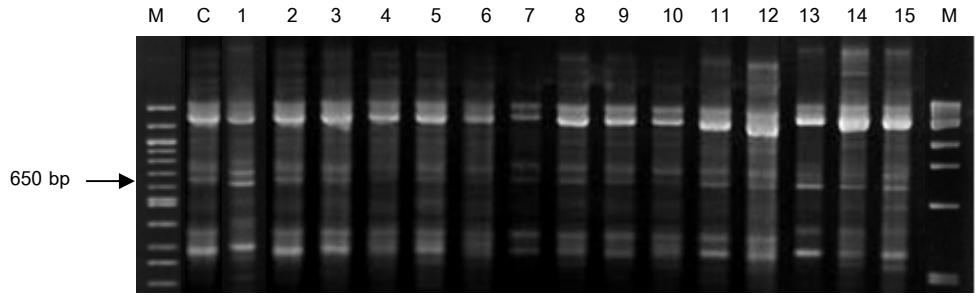


Figure 25 RAPD pattern of somatic embryo lines derived from HMZE obtained with primers OPT06. The amplification products were compared on the basis of molecular size. Lane P and D were profile of DNA fragments from parents. Lane 1-15 were profile of DNA fragments from hybrids.

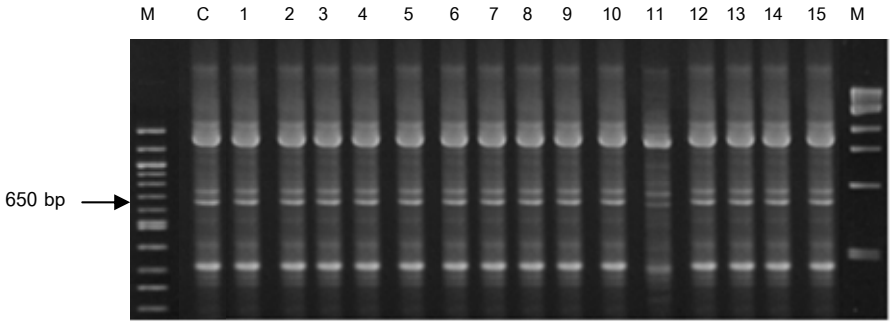


Figure 26 RAPD pattern of regenerated plantlets lines derived from HMZE obtained with primers OPT06. The amplification products were compared on the basis of molecular size. Lane P and D were profile of DNA fragments from parents. Lane 1-15 were profile of DNA fragments from hybrids.

Assessment of somaclonal variation by SSR analysis

For SSR analysis of somatic embryo at globular stage, uniformity assessment was performed with somatic embryo at globular stage lines of cross #77 and cross #137 with primer EgCIR0008. All somatic embryos at globular stage of those two crosses provided no variation of DNA profiles detected by SSR together with primers EgCIR0008 (Figure 27A and 27B). Regenerated plantlets from somatic embryo of cross #118 and cross #119 were uniformity with EgCIR0008 primer. Only little change has been found among somatic embryo at globular stage which was calculated to be 2.4 and 0.8 percent (data not shown) (Figure 27C and 27D). In case of primer EgCIR1772, similar results were obtained from cross #58 and cross #118. Somatic embryo at globular stage resulted in somaclonal variation at very low percentage of 1 (data not shown) indicated by changing of DNA pattern (Figure 28A). Contrary to cross #130, no genetic variation was detected with primer EgCIR1772. The somatic embryos at globular stage of this cross was completely uniform (Figure 28B).

For SSR analysis of regenerated plantlets, uniformity of plantlets with the same pattern of DNA profile was obtained with regenerated plantlets lines of cross #77 and cross #137 with primer EgCIR0008. All regenerated plantlets obtained from both crosses had no variation of DNA detected by SSR profile with primers EgCIR0008 (Figure 29A and 29B). Contrary to regenerated plantlets obtained from cross #58, one unique band was detected for uniformity with EgCIR1772 primer. The change of allele among somclones which was calculated as percentage of somaclonal variation was approximately 1 (data not shown) (Figure 30A). For regenerated plantlets of cross #130, the result revealed that there was no somaclonal variantion detected by this technique (Figure 30B). For cross #118 and cross #119 plantlet regeneration was not obtained. So, the results of somaclonal variation were not detected. However, from the above results of the other crosses it should expect that all regenerated plantlets are true-to-type.

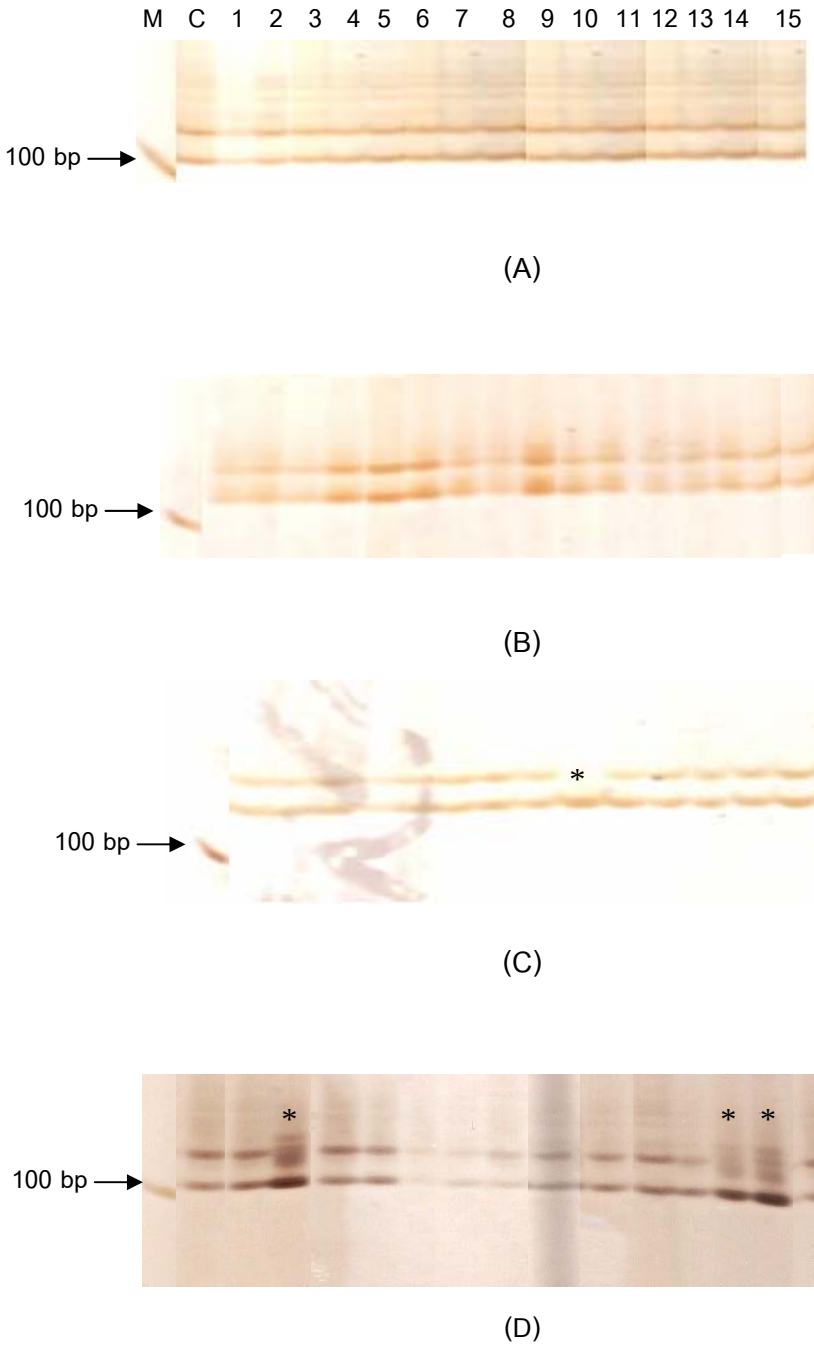
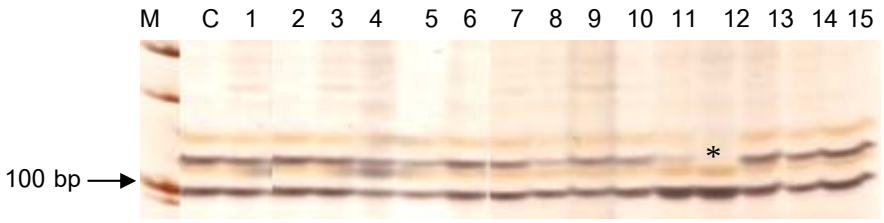


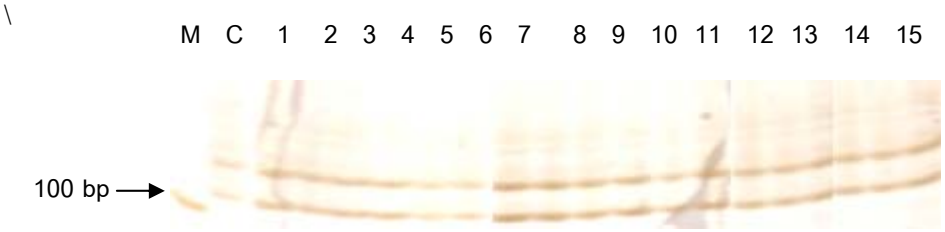
Figure 27 SSR patterns of somatic embryo at globular stage lines of cross #77 (A), cross #137 (B), cross #119 (C) and cross #118 (D) obtained with primer EgCIR0008. The amplification products were compared on the basis of molecular size. Lane 1-15 were fragment from individual somatic embryo at globular stage. The symbol (*) denoted those line that might be somaclonal variation.



(A)

(B)

Figure 28 SSR patterns of regenerated plantlets of cross #58 (A) and cross #130 (B) obtained with primer EgCIR1772. The amplification products were compared on the basis of molecular size. Lane 1-15 were fragment from individual regenerated plantlets. The symbol (*) denoted this line that might be somaclonal variation.



(A)

(B)

Figure 29 SSR patterns of regenerated plantlets from cross #77 (A) and cross #137 (B) obtained with primer EgCIR0008. The amplification products were compared on the basis of molecular size. Lane 1-15 were fragment from individual regenerated plantlets.

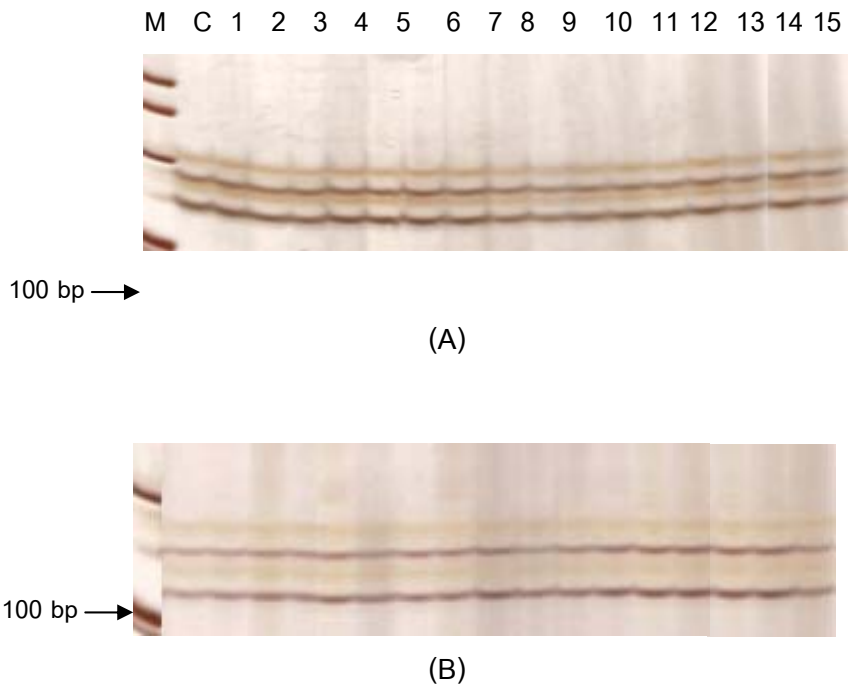


Figure 30 SSR patterns of individual somatic embryo at globular stage from cross #58 (A) and cross #130 (B) obtained with primer EgCIR1772. The amplification products were compared on the basis of molecular size. Lane 1-15 were DNA profiles from individual somatic embryo at globular stage.

Discussion

RAPD marker permits the rapid and cost-effective detection of polymorphism and genetic markers in a variety of plants and animals (Corniquel and Mercier, 1994). Its ability to detect highly variable regions of DNA has a potential application in gene mapping, pedigree analysis and detection of somaclonal variation of hybrid (Parisa *et al.*, 2006). In oil palm, RAPD analysis was used to examine genetic relationship (Moretzsohn *et al.*, 2004) and perform gene linkage (Moretzhn *et al.*, 2000). In this study, RAPD analysis was employed to investigate variation of F1-hybrid derived from somatic embryogenesis. From the present study it is clear that somaclones obtained from our protocol was uniform according to RAPD marker. Using RAPD technique, various authors have reported the absence of genetic variation in potato (*Solanum tuberosum*) (Naoki *et al*., 1994), *Festuca pratensis* (Vallea *et al.*, 1993) and *Pinus thunburghii* (Goto *et al*., 1998). Our results suggest that RAPD technique can be successfully used to assess genetic variations in micropropagated plants. It also demonstrates that genetic integrity of micropropagated plants should invariably be confirmed before transferring plants to field. Our result suggests that amplification bands from the nuclear genome must be used. Polymorphisms suitable for confirmation of hybridity are easily acquired. However, weak bands were sometimes difficult to visualize in the case of somatic hybrids and were not suitable to use in routine analysis. Devos and Gale (1992) reported that RAPD marker was sensitive to the conditions under which the analysis is conducted, may create problems with reproducing experiments under conditions of another laboratory. The reproducibility of the pattern was verified in this study: the primers that generated bands specific to the male parent were repeated at least two more times with the male, the female and the hybrid. Those primers giving the same pattern in the two replicates were chosen. In conclusion, RAPD markers were satisfy the conditions required for confirmation of hybrid in the cross #77 obtained from the parent 366 (Dura) × 172 (Pisifera) and RAPD analysis provides an efficient method for such confirmation.

Assessment of somaclonal variation by SSR analysis

The basic objective of micropropagation is to produce the true-to-type plants, therefore, it is important to certify and retain the fidelity of produced regenerants. SSR analyse have been used to assess the genetic fidelity of micropropagated regenerants. These molecular technique bypass the reliance on diagnostic morphological and phytochemical traits that take time to collect in mass propagation system and assure the fidelity of micropropagated plants (Polanco and Ruiz, 2002; 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). In this study, SSR analysis was employed to investigate variation of F1-hybrid derived from somatic embryogenesis. From the present study it is clear that somaclones obtained from our protocol was uniform according to SSR marker. Using SSR technique, various authors have reported the absence of genetic variation in *Vitis vinifera* (Vignani *et al*., 1996), bread wheat (Akkaya and Buyukunal, 2004), pepper (*Capisum annuum* L.) (Kwon *et al*., 2005), tea (*Camellia* spp.) (Borchetia *et al*., 2009). The absence of any signs of somaclonal varation suggests that our secondary embryogenesis system did not induce changes in gene structure, which had remained stable throughout the 1.5-2 years that all lines had been maintained *in vitro*. We observed no genetic alteration caused by the conversion of somatic embryos into plantlets from cross #58. Similar findings have been reported for *Picea abies* (Heinze and Schmidt, 1994) and *Quercus serrata* (Ishii *et al*., 1999). In addition, clones originated from zygotic embryos of *Quercus robur* were maintained by secondary embryogenesis found that the conversion into plants caused no change in ploidy (Endemann *et al*., 2001). In contrast, plants regenerated from somatic embryos obtained from embryogenic callus cultures have been reported to exhibit somaclonal variation in white spruce (Isabel *et al*., 1996), peach (Hashmi *et al*., 1997) and date palm (Saker *et al*., 2000). Heinze and Schmidt (1994) suggested that callus proliferation probably involves fewer active genes than the formation of somatic embryos or plant regeneration, and since only mutations of active genes cause loss of viability, it is,

therefore, not subject to the same high selection pressure as the latter processes (Shenoy and Vasil 1992).

However, our results found that some cross combination showed the presence of one unique band in the somatic embryo at globular stage, which indicated that only one to two of the somatic embryo at globular stage were different from other *in vitro* raised clones and the mother plant. The occurrence of the same band in independent regenerants has also been observed in rice (Chowdari *et al*., 1998), wheat (Brown *et al*., 1993), sugarcane (Oropeza *et al*., 1995), and rye (Linacero and Vazquez, 1992). The produced somaclonal variants in the present study may be indicative of hypervariable loci, specific regions of a gene or epigenetic influence in the genome. It seems possible that the somaclonal variation induced by tissue culture, in some instances, resemble somatic mutations that occur in nature which lead to the formation of different oil palm clones. Goto *et al*. (1998) suggested that the presence of variations during *in vitro* propagation depends upon the source of explants and method of regeneration. The optimal levels of plant growth substances, especially synthetic hormone, have also been associated with somaclonal variation (Martin and Pachathundkandi, 2006). Even at optimal levels, long-term multiplication may lead to somaclonal or epigenetic variations in micropropagated plants. In conclusion, we have found that most secondary somatic embryos are genetically uniformity detection by SSR analysis in this study case.

CHAPTER 5

Summary

CHAPTER 5

SUMMARY

1. Propagation of Hybrid Oil Palms by Culturing of Zygotic Embryos

MZEs of six genotypes were cultured on MS medium supplemented with various kinds and concentrations of auxins. The result found that kinds and concentrations of auxins containing in culture media had significantly affect on type of calluses. Dicamba provided a nodular callus whereas 2,4-D gave both a white friable callus and a white elongate soft callus. For NAA, it could not induce callus formation. In the present study three different synthetic auxins (NAA, 2,4-D and dicamba) were compared with genotype. Among genotypes tested, cross #58 gave the highest nodular callus at 48.50% and average number of nodule at 18.78±17.49 nodule/callus, significant difference ($\rho < 0.05$) to others genotypes when cultured on 2.50 mg/l dicamba containing medium. For histological study, it was found that 2, 4-D containing medium induced nodular structure from epidermal cells of MZE while dicamba induced from both the epidermis and vascular tissue. In case of NAA containing medium, nodular and elongate root-like structures arose from the epidermal layer of MZE and those structures developed into root primordial.

After 6 weeks of subculture onto MS medium supplemented with 1 mg/l dicamda and 200 mg/l ascorbic acid, all genotypes were promoted to form embryogenic callus. The genotypes and phytohormone played significant role on the percentage of embryogenic callus after 3 months of culture. Cross #58 was identified as the best genotype and produced the highest percentage of embryogenic callus (50.65), significantly different other genotypes after 3 months of culture. For SECI, it was showed that cross #58 gave the highest SECI (11.72), significantly different ($\rho < 0.05$) other genotypes. In case of number of somatic embryo, all genotypes were promoted to form somatic embryos form embryogenic callus when cultured on MS supplemented with 1 mg/l dicamba and 200 mg/l ascorbic acid. Genotypes and hormone used significantly

different affected the percentage of somatic callus and average number of somatic callus formation after 3 months of culture. Cross #58 was identified as the best genotype which produced the highest somatic embryo at GS (17.32%), HS (7.92%), average number of GEs (13.38 ± 3.65) and average number of HEs (4.00 ± 1.33) significantly different other genotypes after 3 months of culture.

All genotypes were promoted to form SSEs from PHEs when cultured on MS medium supplemented with 0.2 M sorbitol and 200 mg/l ascorbic acid for 4 months. Genotype and PGR played significant role in the percentage of SSE formation and average number of SSEs after 4 months of culture. Cross #58 was identified as the best genotype and produced the highest SSE (36%) and average number of SSEs (21.56 ± 14.22), significantly different ($\rho < 0.05$) to other genotypes after 3 months of culture.

2. Screening and detection of hybrids oil palm by DNA markers

RAPD marker was employed to verify six crosses of hybrids oil palm using HZMEs. The result of our experiment indicated that RAPD marker can be used for identification of hybrid. Only one primer, OPT06, out of 7 primers provided clearly DNA pattern. Cross #77 and cross #119 showed specific fragment at 650 and 400 bps with OPT06 primer, respectively. For SSR marker, EgCIR008 and EgCIR1772 primers proved to be suitable for verification of six crosses of hybrid oil palm. EgCIR008 could identified four cross combination while EgCIR1772 primer was able to identify two different hybrid combinations. Cross #77 showed MPS band at 115 and 120 bps and FPS band at 105 bps with EgCIR008 primer. Cross #118 was heterozygous with primer mEgCIR008 showing both of MPS band at 115 bps and FPS at 105 bps. Cross #119 was also heterozygous with primer mEgCIR008 showing both of MPS band at 115 bps and FPS at 105 bps. For cross #137 showed MPS band at 115 and 120 bps and FPS band at 105 bps with EgCIR008 primer. In case of primer EgCIR1772 generated both of MPS band at 110 and 115 bps and FPS band at 105 and 108 bps with the cross #58. Cross #130 was

also heterozygous with primer mEgCIR1772 showing both of MPS band at 115 bps and FPS at 105 bps.

Assessment of somaclonal variation was to detect uniformity of somatic embryos at GS, regenerated plantlet and their mothernal plants of cross #77 by RAPD marker. It was clear that somaclones obtained from our protocol was uniform and successfully used to assess genetic variations in micropropagated plants. In addition, all somatic embryos at GS and regenerated plantlets of cross #77 and cross #137 provided no genetic variation of DNA profiles detected by SSR analysis with primers EgCIR0008. For cross #130, no genetic variation was detected with primer EgCIR1772. The somatic embryos at GS of this cross were completely uniform. However, only little change has been found in some somatic embryo at GS of cross #118 and cross #119 which was calculated to be 2.4 and 0.8 % only (data not shown). In case of cross #58, some somatic embryo at GS produced somaclonal variation at very low percentage of 1 (data not shown) indicated by changing of DNA pattern. However, regenerated plantlets developed from those somatic embryos were not genetic variation.

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APPENDIX

Appendix

Preparation of solution buffers and reagents

1. DNA isolation by the modified CTAB method

1.1 CTAB (Hexadecyl trimethyl-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 ddH_2O. Add 2 g of CTAB and put in the water bath at 60°C for 15-20 minutes to dissolve the CTAB (Don't shake the solution – the detergent will bubble up too much). Sterilize using an autoclave. Prior to starting extraction, add 2% β-mercaptoethanol in the buffer. Once these have been added the shelf life of the buffer is only 2-3 days.

1.2 1.0 M Tris-HCl (pH 8.0)

- 121.1 g of Tris-HCl

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

1.3 TE buffer

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

Adjust the volume to 500 ml with distilled water and sterilize using an autoclave.

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 using an autoclave. 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 using an autoclave. To make 0.5X TBE buffer before using.

2. DNA isolation by the modified Te-chato method

2.1 TE buffer

- 500 µl of 20 mM Tris-HCl (pH 8.0)
- 200 µl of 0.1M EDTA (pH 8.0)

Adjust the volume to 500 ml with distilled water and sterilize using an autoclave.

2.2 10% SDS

- 5 g of SDS

Adjust the volume to 50 ml with distilled water and sterilize using an autoclave.

2.3 5M Ammonium acetate

- 38.54 g of ammonium acetate

Adjust the volume to 50 ml with distilled water and sterilize using millipore filter

2.4 0.5 M EDTA

- 37.224 g EDTA

Add about 700 ml H_2O and add 16-18 g of NaOH pellets for adjust pH to 8.0 by with a few more pellets, EDTA won't dissolve until the pH is near 8.0. Bring total volume to 1 L with ddH_2O.

Appendix table 1 Composition of nutrition of Murashige and Skoog (MS)

Compositions	Volume (mg/l)
Major 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
Minor 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 elements	
Myo-inositol	100.000
Nicotinic acid	0.500
Pyridoxine HCl	0.500
Thaiamine HCl	0.100
Glycine	2.000
Sucrose (g)	30.000
Agar (g)	7.500
pH	5.7

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List of Publications and Proceedings

Thawaro S. and S. Te-chato. 2007. Auxins as effect type of callus formation from mature zygotic embryo culture of hybrid oil palms. International conference on integration of science and technology for sustainable development, Bangkok, Thailand. 26-27 April 2007. pp. 246-250. (Oral presentation)

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