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*Original Article*

# Verification of legitimate *tenera* oil palm hybrids using SSR and propagation of hybrids by somatic embryogenesis

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**Abstract**

Oil palm planting material consists solely of *tenera* hybrids, originating from crosses between *dura* and *pisifera* types. To confirm the parentage of the *tenera* hybrids, we used a DNA molecular marker approach based on simple sequence repeat (SSR). 6 parental lines of *pisifera* and 2 of *dura* were used to produce 6 independent progenies. SSR markers were tested (8 SSR primers) to valid the progenies from crosses #77 [366 (D) × 172 (P)], #58 [366 (D) × 72 (P)], #118 [366 (D) × 206 (P)], #119 [865 (D) × 206 (P)], #130 [865 (D) × 110 (P)], and #137 [366 (D) × 777 (P)]. All primers tested could amplify parental DNA. Secondly, half mature zygotic embryos consisting of the coleoptile region of those combinations was cultured on Murashige and Skoog (MS) medium plus various kinds and concentrations of auxins for callus induction. The results revealed that primers mEgCIR008 provided a clear DNA pattern and could be used for hybrid verification of the crosses 366 (D) × 172 (P). The highest frequency of nodular callus formation at 65% was obtained on MS medium supplemented with 2.5 mg/l 3, 6-dichloro-o-anisic acid (dicamba), significantly superior to the other kinds and concentrations of auxins. Moreover, somatic embryo formations at the globular and haustorium stages were achieved at 7.17% and 4.59%, respectively when cultured on MS medium supplemented with 0.2 M of sorbitol and 200 mg/l ascorbic acid for 3 months.

**Keywords:** hybrid verification, somatic embryogenesis, *Elaeis gunieensis*, SSR marker oil palm

## 1. Introduction

Oil palm (*Elaeis guineensis* Jacq.) is a diploid monocotyledon belonging to the family Arecaceae. It is an economically important product, as it is the source of palm oil, the most traded vegetable oil in the international market, and it is increasingly used in the food industry (Corley and Tinker, 2003). The large amount of oil produced in the oil palm fruit is a unique biological characteristic of this palm species (Hartley, 1977). In Thailand, the government aims to increase palm oil production in order to serve the biodiesel industry, due to a sharp rise in global oil prices, which necessitates the finding of alternative energy sources. Due to a continuous rise in demand for biodiesel, it was estimated that the domestic consumption of biodiesel will grow to 31.3 billion liters per year in the next six years. By the year 2012, the area needed to cultivate oil palm will be 9.1 million hectares (Watcharas, 2008). All modern commercial palms are hybrid thin-shelled *tenera* palms, which were chosen for their high oil productivity containing a high proportion of unsaturated fatty acids. However, the tenera hybrids show a very high variation in oil yield with the best plants yielding 40% higher than the non-tenera average. As an oil palm is normally propagated by seed, a high variation is found in the field and it takes a long time to perform trials using available sustainable genetic trait production through traditional breeding. In order to overcome this limitation, nowadays plant micropropagation is used for plant breeding, and the clonal

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propagation of oil palms through tissue culture is common (Aberlenc-Bertossi *et al.*, 1999; Rajesh *et al.*, 2003). However, a shortage of good hybrid oil palms is a major problem of oil palm cultivation in Thailand. To optimize time and costs of the planting, it is necessary to verify the hybridity of new seedlings at an early stage, before commercial plantation, in order to assure uniformity and stability of the field performance and yield. DNA molecular marker technology, which is based on sequence variation of specific genomic regions, provides a powerful tool for hybrid identification and seed verification with the advantages of time-saving, less labor consumption, and more efficiency (Hu and Quiros, 1991; Mongkolporn *et al.*, 2004; Dongre and Parkhi, 2005; Liu *et al.*, 2007; Garg *et al.*, 2006). At present, the main DNA marker techniques are Restriction Fragment Length Polymorphism (RFLP), Random Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP), and Simple Sequence Repeat (SSR). SSR has much more polymorphism than most the other major DNA markers, and is co-dominant of multiallelic nature, and large in quantity (Wang *et al.*, 2002). Therefore, SSR has become an ideal molecular marker in plant gene mapping, genetic analysis, and marker-assisted plant improvement programmes (McCouch *et al.,* 2002). It permits identification of DNA polymorphisms and can be used to amplify particular fragments of genomic DNA (Bielawski *et al.*, 1996) for various purposes for example, DNA fragment profiles have been employed to analyze the genetic relationships of plant species (Ayana *et al.*, 2002), and in *Elaeis*, SSR markers have been employed for gene mapping (Billotte *et al.*, 2005; Singh *et al.*, 2007). In the present paper, we describe verifying half seeds of six combinations of hybrid using half-seed by SSR technique. The remaining half seeds were brought to induction of callus for micropropagation. The nodular callus is in reference to the work of Te-chato *et al.* (2003). The hybrid crosses were screened and commercially propagated for high oil yield producing clones.

## 2. Materials and Methods

### 2.1 Plant material

Mature fruits of oil palms from six hybrids, crosses #77 [366 (D) × 172 (P)], #58 [366 (D) × 72 (P)], #118 [366 (D) × 206 (P)], #119 [865 (D) × 206 (P)], #130 [865 (D) × 110 (P)], and #137 [366 (D) × 777 (P)], were kindly provided by Dr. Theera Eksomtramage of the Agricultural Research Station, Klong Hoi Khong, Hat Yai, Songkhla, Thailand. After 180 days of pollination, all seeds were collected and extracted from the fruit, cracked by hammer and trimmed by pruning scissors to remove excess kernel. Mature zygotic embryos embedded by kernel in cubes of 3×3×3 cm 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 a further 20 min. The cubes were then thoroughly washed in sterile water three times, and the embryos excised from the cubes and cultured on media.

### 2.2 Hybrid verification via SSR analysis

A leaf sample (0.05 g.) and half-embryo (15-20 mg) of each genotype were collected and the DNA isolated following the method of Te-chato (2000). SSR analysis of genomic DNA was carried out using 8 microsatellite loci amplified from oil palms. Primers were obtained from Operon Tech. (California, USA) (Table 1). 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,

Table 1. Primers used in SSR analysis of identified hybridity in the parents plant of *Elaeis quineensis* Jacq. and allele size of each primer.

| Primer name | Repeat motif | 5'-3' Forward primer | 5'-3' Reverse primer | Annealing temperature (°C) | allele size | No. of SSR allel | Polymorphic loci (%) |
|---|---|---|---|---|---|---|---|
| EgCIR008 | $(GA)_{17}$ | CGGAAAGAGGGAAGATG | ACCTTGATGATTGATGTGA | 52 | 105-150 | 8 | 11.11 |
| EgCIR0243 | $(GA)_{17}$ | TGGAACTCCTATTTTACTGA | GCCTCGTAATCCTTGTCA | 52 | 248-270 | 6 | 11.11 |
| EgCIR0337 | $(GT)_6$ $(GC)_4$ | GTCTGCTAAAACATCAACTG | GAGGAGGAGGGGAACGATAA | 52 | 145-189 | 1 | 11.11 |
| EgCIR0409 | $(CCG)_6$ | AGGGAATTGGAAGAAAAGAAAG | TCCTGAGCTGGGGTGGTC | 58 | 252-298 | 5 | 33.33 |
| EgCIR0446 | $(CCG)_7$ | CCCCTTCGAATCCACTAT | CAAATCCGACAAATCAAC | 52 | 202-275 | 3 | 11.11 |
| EgCIR0465 | $(CCG)_6$ | TCCCCCACGACCCATTC | GGCAGGAGAGGCAGCATTC | 52 | 125-197 | 5 | 33.33 |
| EgCIR0781 | $(GA)_{17}$ | CCCCTCCCTACCACGTTCCA | TGTTTGCTGTTGCTCTTTGATTTTC | 52 | 207-288 | 7 | 11.11 |
| EgCIR0905 | $(GT)_{14}$ ctca$(GA)_{11}$ | CACCACATGAAGCAAGCAGT | CCTACCACAACCCCAGTCTC | 52 | 231-284 | 4 | 11.11 |
| Average | | | | | | 4.86 | 16.67 |
| SD | | | | | | 2.23 | 10.29 |

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 analyzed on 6% (w/v) denaturing polyacrylamide gels. Silver staining was conducted according to the protocol of Benbouza *et al.* (2006).

### 2.3 Effect of various kinds and concentrations of auxins on type of callus formation

The remaining second half mature zygotic embryos (HMZEs) consisting of shoot apical tissues were inoculated in culture tubes containing 10-15 ml of modified MS medium supplemented with either 215.05 μM NAA or 2, 4-D or dicamba at concentrations of 11.31 and 22.62 μM for callus induction. All media were solidified with 0.75% agar. The pH of all culture media was adjusted to 5.7 with 0.1 N KOH before adding agar and autoclaving at 1.05 kg/cm$^2$, 121°C for 15 min. The cultures were placed under light conditions (3,000 lux illumination for 16 hrs photoperiod) at 25±2°C and subcultured every 4 months on the same medium component for 3 months. The percentage of cultures that produced callus, the type of callus, and number of embryos per tube were recorded after 1-3 months of culture by counting under a stereo-microscope (Nikon, SMZU).

### 2.4 Proliferation of embryogenic callus (EC) and formation of somatic embryos (SE)

Nodular calluses (NCs) (approximately 5 nodules/explant) of the cross 366 (D) × 172 (P) were carefully separated and inoculated on MS medium supplemented with 4.52 μM diacamba, 200 mg/l ascorbic acid, 3% sucrose and gelling with 0.75% agar-agar. The culture was maintained under the same conditions as described in the first experiment and subcultured at monthly intervals. The percentage of embryogenic callus and average number of somatic embryos per tube was recorded after 1 month of culture. The data were recorded for 3 months.

### 2.5 Statistical analysis

For experimental design and statistical analysis, a completely randomized design (CRD) with four replicates (each replicate consist of 10 embryos) was performed. Data were analyzed using analysis of variance (ANOVA).

## 3. Results

### 3.1 Hybrid verification via SSR analysis

A set of 8 SSR primers (EgCIR008, EgCIR0243, EgCIR0337, EgCIR0409, EgCIR0446, EgCIR0465, EgCIR 0781, and EgCIR0905) 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 4.86±2.23 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 1). The results revealed that only one primer, EgCIR008, provided clearly DNA pattern and produced codominant marker (Figure 1 and 2). Primer mEgCIR008 showed the greatest capacity for distinguishing polymorphic fragments in the half-embryo cultures (Figure 3). Cross #77 obtained from the parent 366 (Dura) × 172 (Pisifera) was a good model for the verification of hybrids. The results from DNA pattern among cross #77 showed specific fragment and could be used to distinguish hybrid between dura and pisifera. All hybrids showed the DNA patterns between the two parents and more additive bands according to SSR analysis. 75 out of 100 plants were heterozygous with primer mEgCIR008 showing the presence of both male and female fragments of DNA. Six hybrids were found to be homozygous of the male parent allele, while only one was homozygous with a female specific allele (Figure 4).

### 3.2 Effect of various kinds and concentrations of auxins on type of callus formation

HMZE consisting of a coleoptile of the cross 366 (D) × 172 (P) swelled at 10-14 days of culture and started to form callus at 4-5 weeks of culture. Most auxins promoted callus formation from HMZE of the cross 366 (D) × 172 (P) at week 6 after subculture. Four types of calluses could be distinguished from those cultures friable (FC), compact (CC), nodular (NC), and root-like (RLC) calluses. The FC was yellow, translucent and succulent, the CC was muddy white and compact, the NC was yellow or pale yellow and compact, and the RLC was elongated and white with a soft texture. The kinds and concentrations of auxins used as supplements in the culture medium had a significant effect on the type of callus that resulted. The characteristics of the calluses obtained in media containing NAA, 2, 4-D or dicamba were quite different. Dicamba gave a yellow compact callus (so called NC), while 2, 4-D and NAA gave both a white FC and RLC formation (Figure 5). The highest percentage of nodular callus, 65%, was obtained from 11.31 μM dicamba after 3 months of culture (Table 2).

### 3.3 Proliferation of embryogenic callus (EC) and formation of somatic embryo (SE)

Approximately 5 nodules of NC from the cross 366 (D) × 172 (P) were gradually expanded by increasing the time of culture. After 3 months of culture, MS medium supplemented with 4.52 μM dicamba gave the highest EC proliferation at 7.17% and the highest average number of ECs at 5.33 per explant (Figure 6a). Somatic embryo (SE) formation at the haustorium stage was 4.59% with an average number of HEs at 2.98 per explant when cultured on MS medium

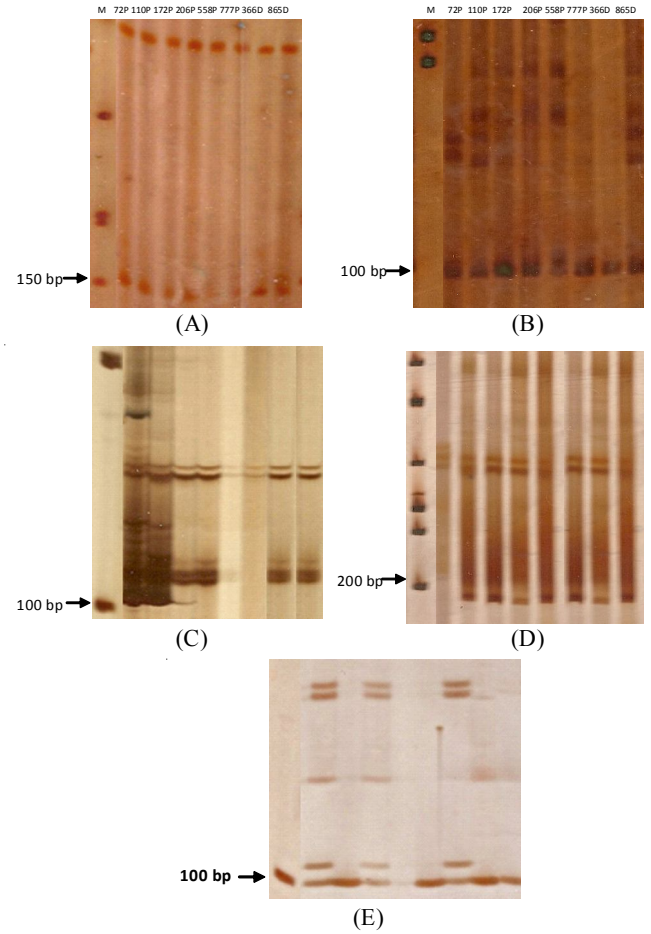


Figure 1. Amplification patterns of SSR locus of parents. The amplification products were compared on the basis of molecular size. Lane 1, 2, 3, 4, 5, and 6 were fragments from pisifera plants. Lane 7 and 8 were fragments from dura plants, obtained with primers EgCIR0337 (A), EgCIR0409 (B), EgCIR0781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

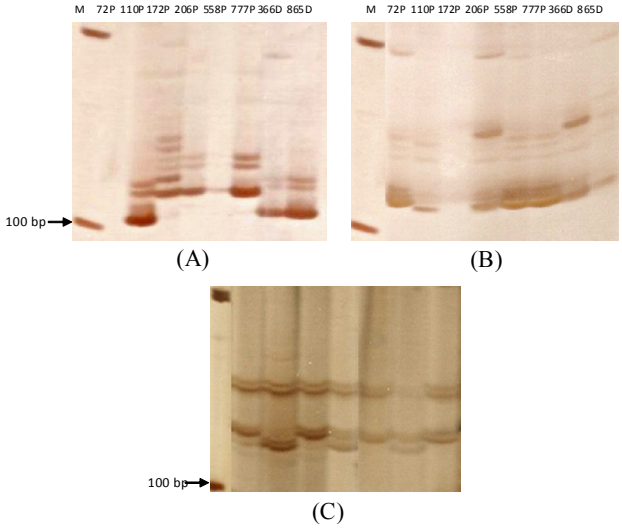


Figure 2. Amplification patterns of SSR locus of parents. The amplification products were compared on the basis of molecular size. Lane 1, 2, 3, 4, 5, and 6 were fragments from pisifera plants. Lane 7 and 8 were fragments from dura plants, obtained with primers EgCIR0008 (A), EgCIR0446 (B), and EgCIR0230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

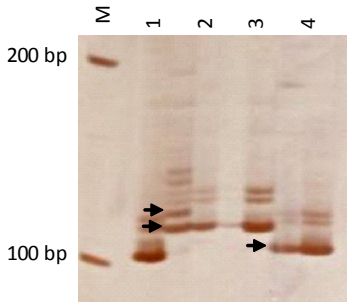


Figure 3. SSR analysis of the parental lines 366 (D) × 172 (P) with primer mEgCIR0008. Lane M: DNA ladder, Lanes 1-6: male parent, and Lanes 7-8: female parent. The arrows indicate male and female parent-specific markers.

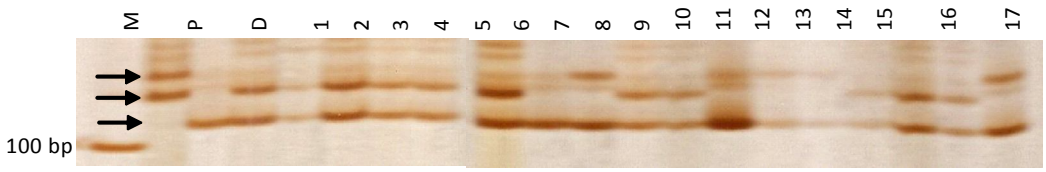


Figure 4. SSR analysis of the parental 366 (D) × 172 (P) with primer mEgCIR0008. Lane M: DNA ladder, Lane P: male parent, Lane D: female parent and Lanes 1-20: oil palm hybrids. The arrows indicate male and female parent-specific markers.

supplemented with 0.2 M of sorbitol and 200 mg/l ascorbic acid for 3 months (Figure 6b, Table 3).

## 4. Discussion

### 4.1 Hybrid verification via SSR analysis

SSR marker was employed to confirm *tenera* hybrid parentage. Among eight primers were identified to be codominant. In the present study, the SSR marker showed complementary banding pattern of both parents. It was valuable to distinguish the F1 from their male and female parents. 75 out of 100 plants were heterozygous with primer mEgCIR 008 showing the presence of both male and female fragments of DNA. Six hybrids were found to be homozygous of the male parent allele, while only one was homozygous with a

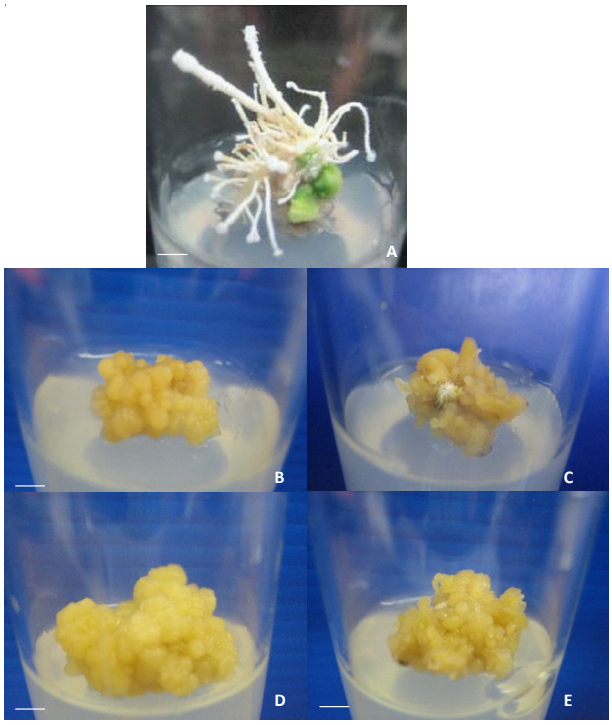


Figure 5. Callus formation from culturing mature zygotic embryo cultured on solid MS supplemented with various hormones for 3 months. Callus cultured on MS medium supplemented with 215.05 µM NAA (A), 11.31 µM 2, 4-D (B), 22.62 µM 2, 4-D (C), 11.31 µM dicamba (D), and 22.62 µM dicamba (E) (bar = 2.50 mm).

Table 2. Effect of various kinds and concentrations of auxin on the type of callus formation from mature zygotic embryos of the cross 366 (D) × 172 (P) after culture for 3 months.

| Auxin | Concentration (µM) | Type of callus formation (%) | | | | Average number of calluses/embryo | |
|---|---|---|---|---|---|---|---|
| | | Friable callus | Compact callus | Nodular callus | Root like callus | Nodular callus ± SD | Root like callus ± SD |
| NAA | 215.05 | 15b | 5 | 2.50d | 25a | 3.82±1.72c | 1.55±0.82a |
| 2,4-D | 11.31 | 22.50b | 7.50 | 27.50b | 10ab | 8.22±4.15bc | 0.44±0.33b |
| | 22.62 | 20b | 7.50 | 35b | 0b | 7.93±4.23bc | 0.00±0.00c |
| Dicamba | 11.31 | 50a | 12.50 | 65a | 0b | 17.31±8.87a | 0.00±0.00c |
| | 22.62 | 10b | 12.50 | 10c | 0b | 11.75±5.06ab | 0.00±0.00c |
| F-test | | * | ns | * | * | * | * |
| C.V. (%) | | 44.48 | 44.42 | 31.02 | 38.17 | 34.55 | 38.50 |

ns: not significant difference
*Significant different at $p \leq 0.05$
Mean followed by the same letter do not different significantly ($p<0.05$).

female specific allele. These results were similar to those reported by Liu *et al.* (2007) in tomato, who successfully used an SSR marker for testing the seed genetic purity of two commercial hybrid tomato cultivars 'Hezuo 903' and 'Sufen No. 8'. Our results showed that SSR markers are an effective tool for the verification and identification of hybridity in oil palms.

### 4.2 Effect of various kinds and concentrations of auxins on type of callus formation

Different genotypes gave the different response on the percentage of cultures producing callus and type of callus formation. Because of genotype influencing, genotypes specificity for callus induction has been reported in numerous

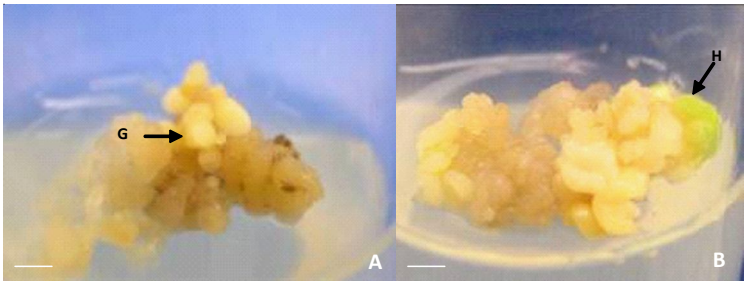


Figure 6. Somatic embryo formation. A: Globular stage (G), and B: haustorium stage (H) after culturing NC on solidified MS medium supplemented with 1 mg/l dicamba and 200 mg/l ascorbic acid for 3 months (bar = 2.50 mm).

Table 3. Effect of time period on somatic embryo formation of the cross 366 (D) × 172 (P) after being cultured on MS medium supplemented with 1 mg/l dicamba and 200 mg/l ascorbic acid for 3 months.

| Time period (months) | Somatic embryo (%) | | Average number of somatic embryos / explant | |
|---|---|---|---|---|
| | Globular stage | Haustorium stage | Globular ± SD | Haustorium ± SD |
| 1 | 0.50c | 0.25 | 0.75±0.15c | 0.33±0.13 |
| 2 | 3.44b | 1.98 | 1.22±1.08b | 0.88±0.44 |
| 3 | 7.17a | 4.59 | 5.33±2.71a | 2.98±0.78 |
| Mean | 3.70 | 2.27 | 2.43±1.31 | 1.40±0.45 |
| F-test | * | ns | * | ns |
| C.V. (%) | 25.71 | 32.44 | 21.38 | 33.51 |

ns: not significant difference
*Significant different at $p \leq 0.05$
Mean followed by the same letter do not different significantly ($p<0.05$).

cultivars: *Feijoa sellowiana* (Guerra *et al.*, 2001), *Carthamus tinctorius* (Mandal *et al.*, 2001) and Rye (*Secale cereale* L.) (Ward and Jordan, 2001). In this study, we found that different genotypes revealed different types of callus formation (data not shown). Cross 58 obtained from 366 (D) × 172 (P) gave the highest nodular callus formation and the average number of nodular callus showed a significant difference to others genotypes. So that 366 (D) × 172 (P) was classified as high capacity in their callus formation, significant different to other genotypes. Similar results were also reported by Sanchez-Romero (2005) in Avocado, Sairam *et al.* (2003) in soybean, El-Bakry (2002) in tomato, Can *et al.* (2004) in perennial ryegrass, and Diana (2002) in coffee. Similar findings are also observed in *in vitro* culturing of mature and immature zygotic embryo of oil palm (Chehmalee and Te-chato, 2007; Chehmalee and Te-chato, 2008; Sanputawong and Te-chato, 2008). Genotypes of the selected explants may have influenced upon the type of responsive callus like the report of Sarasan *et al*. (2005). In this present study it is clearly evident that the genotype plays a role in the type of callus. Moreover, PGRs played an important role on callus formation as well. Gueye *et al.* (2009) suggested that localized endogenous auxin quantities related to polar auxin transport dependent efflux may be necessary for the cellular modifications observed in fascicular parenchyma (FP) cells and/or in perivascular sheath cells (PSCs) that are the origin of callus formation in date palm leaf segments. Different plant/palm species responded to different kinds of auxins, especially, embryogenic callus induction such as *Phoenix dactylifera* (Schwendiman *et al.*, 1988), *Cocos nucifera* (Fernando *et al.*, 2003), or *Bactris gasipaes* (Steinmacher *et al.*, 2007). The results revealed that the highest frequency of callus formation was obtained on MS medium supplemented with 2.50 mg/l dicamba, significant different to other kinds and concentrations of auxins. Maria and Heidi (2002) also reported that dicamba was effective for callus induction from culturing of wheat (*Triticum aestivum* L.). Similar results were also found in immature embryo culture of winter wheat (Carman *et al.*, 1988) and spring wheat cultivars (Hunsinguer and Schauz, 1987). Dicamba is a promising auxins, which has been reported to be an effective on promoting direct and indirect embryogenic callus induction from cultured mature zygotic embryo and young of leaf oil palm (Te-chato, 1998a). Time used for callus induction in culture medium supplemented

with dicamba was shorter than 2,4-D and NAA. A similar result was obtained from culturing young leaves of the same plant (Te-chato, 1998b; Te-chato *et al.*, 2003). Among the three auxins tested, dicamba was the best for induction of callus, in a manner previously confirm in spring barley (*Hordeum vulgare* L.), in winter barley (Halamkova *et al.*, 2004), and in *Areca catechu* (Wang *et al.*, 2006). In this present study it is clearly evident that both genotypes and various kinds and concentrations of auxins play a role in the type of callus.

### 4.3 Proliferation of embryogenic callus (EC) and formation of somatic embryo (SE)

In the present study, the percentage of EC formation from the cross 366(D) × 172(P) was gradually increased by increasing the time of culture. After three months of culture, MS medium supplemented with 1 mg/l dicamba gave the best response on EC proliferation. The highest SE formation also was obtained from MS medium supplemented with 1 mg/l dicamba (data not shown). Dicamba was found to be the best auxin for *in vitro* mass propagation of both seedling and mature oil palm (Te-chato *et al*., 2003). In addition, embryoids developed on medium containing 1 mg/l dicamba were found to be superior in inducing early stage of embryoid subsequent to further development of mature or haustorium embryoids (Te-chato, 1998). Some authors reported that a low concentration of dicamba promoted somatic embryogenesis from immature inflorescence (Steinmacher *et al*., 2007). A similar result was obtained in callus culture of *Areca catechu*. A decrease in the concentration of dicamba stimulated the proliferation rate of EC and also promoted a large number of embryoid formation (Wang *et al*., 2006).

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