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Assessment genetic instability of regenerated plantlets from long-term culture of oil palm through SSE formation by SSR marker

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Verification of somaclonal variation of *in vitro* oil palm plantlets from tissue culture technique maintained on MS solidified medium with a low concentration of plant growth regulators (PGR) for more than 10 years under a 14 h photoperiod at 28 ± 2 °C were investigated. Un-emerged young leaves were cultured on MS medium supplemented with 3% sucrose, 200 mg/l ascorbic acid, 2.5 mg/l dicamba and 0.6% agar in the dark for 56 days. Primary calli obtained from leaf segments were transferred to embryogenic callus induction medium (MS medium supplemented with 3% sucrose, 200 mg/l ascorbic acid, 0.1-0.5 mg/l dicamba and 0.6% agar) and sub-cultured to fresh medium at 4-week intervals. Within 10 year of culture, various stages of somatic embryos from globular to haustorium (mature somatic embryo) were obtained. In some cultures, haustorium embryos (HEs) developed from embryogenic callus after 3 weeks of subculture. Basal MS medium supplemented with 0.1 mg/l dicamba in the presence of 0.2M sorbitol resulted in the best SSE induction both percentage (100) and number of SSE (21.55 SSEs/HE). SEE could germinate on PGR-free medium at the highest percentage of 78 and gave rise to small plantlets within 30-45 days. DNA was extracted from secondary somatic embryos (SSEs)-derived plantlets using a modified CTAB protocol (Doyle and Doyle, 1990). Assessment of regenerated plantlets was carried out using SSR technique with 9 primers. The results revealed that primer EgCIR0008 provided clear DNA patterns with monomorphism bands. By this primer, it is clear evident that there was no somaclonal variation found from tissue-culture-derived plantlets of oil palm. Therefore, propagation of oil palm through tissue culture technique under our protocol gave uniformity of plantlets.

Key words: Oil palm, plantlet, somaclonal variation, SSR

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Introduction

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). 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, 2008). 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 (Promchan and Te-chato, 2007).

Type, concentration, and combination of PGRs in the culture media can greatly affect callus formation of oil palm tissue culture. Teixeira *et al.* (1993; 1994; 1995) determined that 2,4-D in the initiation media was essential for callus and cell suspension induction from mature zygotic embryos (MZEs) and the optimal level was 500 μM. Te-chato *et al*. (1998b) 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%. 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). Te-chato (2001) reported that dicamba was superior to other auxins whereas NAA gave the poorest result in callus formation. Low concentration of dicamba at 2.5 mg/l in culture medium has been reported to be effective for primary callus induction from both zygotic embryos and young leaves (Te-chato *et al*., 2003; Chehmalee and Te-chato, 2008). Moreover, dicamba has been reported to be an effective auxin for both shortening time period for callus induction and increasing a large number of somatic embryo (Te-chato *et al*., 2004). First lot of vitro-plants from embryogenic callus maintained in *in vitro* conditions for five years produced normal plants and bearing fruits after 2-3 years of transferring in the field.

Recently, molecular marker techniques based on the polymerase chain reaction (PCR) has been one of the most commonly used for characterization of genetic variability, genotype identification, genetic instability analyses and selection and breeding purposes (Tingey and Tufo, 1993). Among those techniques simple sequence repeat (SSR) was proved to be very commonly used in identification hybridity of oil palm F1 hybrid (Thawaro and Te-chato,

2009), verification of uniformity of oil palm plantlets from tissue culture (Sanputawong, 2010). To make sure that long-term culture of embryogenic callus of elite oil palm clone can produce uniform plantlets marker assist selection (MAS) is necessary. Thus, in this study, plantlet regeneration from long-term maintenance of embryogenic callus for more than 10 years will be discussed and assessment of genetic instability using SSR is described.

Materials and methods

Plant material

Elite clone of oil palm cultivar tenera (DxP) were selected from Thepa Research Station, Faculty of Natural Resources, Prince of Songkla University. The plant was ten years old, free from diseases with high sex-ratio, fresh fruit bunch and oil yield (oil extracted ratio; OER). Shoot of the plant was cut, trimmed to desirable size and transferred to laboratory for excision of young leaves to use as explants. The leaves were surface sterilized according to a technique described by Te-chato *et al*. (1988). The leaves were used as plant material for callus induction.

Primary callus induction

For callus induction, un-emerged young leaves were excised, sterilized, cut into 3x3 mm^2 fragments and cultured on Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with 3% sucrose, 200 mg/l ascorbic acid and 2.5 mg/l dicamba (designated MS-I medium). The medium was solidified with 0.6% agar-agar (commercial grade). The cultures were initially maintained in the dark for 45-120 days at 28±0.5^{o}C. Primary calli obtained from leaf segments at various periods during the 56 days (or 8 weeks) of culture were transferred to embryogenic callus induction medium.

Embryogenic callus induction

Primary callus induced from leaf fragments was separated and transferred to fresh medium with the same components except the concentration of dicamba which were decreased to 0.1-1.0 mg/l and supplemented with 200 mg/l ascorbic acid (designated MS-P medium). In order to proliferate or multiply the callus MS-P medium was used for further culture or subculture. The cultures were placed under light conditions (25 µmol/m^2/sec, 14 hour photoperiod) and subcultured to MS-P medium at 4-week intervals for more than ten years.

Induction of secondary somatic embryogenesis (SSE)

Fully mature developed embryos, so called haustorium embryo (HE), were carefully detached and inoculated on PGR-free MS medium supplemented with sorbitol 0.1, 0.2 or 0.3M and gelled with 0.65% agar-agar (Bacteriological grade, Hi-media). The pH of the medium was adjusted to 5.8 prior to autoclaving. Cultures were maintained at 25µmol/m^2/sec (14 hour photoperiod) at a temperature of 28±0.5°C. HEs were uniformly spread on the surface of the medium. There were 4 replicates (Borosil culture tubes of 25×150 mm containing 10 ml of medium) each containing 25 HEs per replicate. The percentage of cultures that produced secondary somatic embryos (SSEs) and number of SSE per tube were recorded after 12 weeks of culture by counting under a stereo-zoom microscope (Nikon, SMZU).

Germination of SSEs

SSEs together with haustorium-staged embryos from sorbitol containing medium were transferred to PGR-free MS medium supplemented with 3% sucrose, solidified with 0.7% agar and adjusted to pH 5.7 before autoclaving. All cultures were carried out in bottle (60x110 mm containing 25 ml of medium) and maintained at 28±0.5°C under 14 h photoperiod, 25µmol/m^2/sec illumination. After 8 weeks (observed at weekly intervals) germination percentage and number of seedlings/SSE clump were recorded.

Assessment of genetic instability of long-term regenerated plantlets via SSR marker

Young first and second leaves of 20 plantlets at approximately 200 gram fresh weight (gFW) were collected and subjected to extract DNA according to the method described by Doyle and Doyle (1990) with some modification. In brief, the leaves were ground in liquid nitrogen and powder of leaf grinding was added with CTAB buffer in the presence of β-mercaptoethanol at quantity of 700 µl and crushed firmly. The mixture of powder and extraction buffer was transferred to 1.5 ml micro-centrifuge tube, incubated at 60^{o}C for 60 min (swirl the tube every 10 min), added 800 µl of chloroform and centrifuged at 12000 rpm for 15 min. Supernatant was removed to new tube, added with 750 µl isopropanol and gently swirl the tube to precipitate DNA. Isolated DNA was precipitated by centrifugation at 12000 rpm for 5 min, air-drying and finally dissolving in 20 µl TE buffer [10 mM Tris – HCl (pH 7.5) and 1 mM Na2EDTA (pH 7.0)].

SSR analysis of genomic DNA was carried out using 9 microsatellite primers (EgCIR0008, EgCIR0234, EgCIR0337, EgCIR0409, EgCIR0446, EgCIR0465, EgCIR0781, EgCIR0905 and EgCIR1772) as described by Billotte *et al*. (2001). Amplification of genomic DNA was done in a 10 µl mixture containing 2.5 mM $MgCl_2$, 10x*Tag* buffer, 100 µl of each dNTP, 0.5 mM of each primer, 1.5 units of *Tag* polymerase and 20 ng of template DNA (Billotte *et al*., 2005). PCR amplifications were carried out on a thermo cycler (TC-XP-G, Japan) using the following program: denaturation at 95^{o}C for 1 min, 35 cycles of 94 ^{o}C for 30 s, 52 ^{o}C for 60s, 72 ^{o}C for 120s, and a final elongation step at 72^{o}C for 8 min. An equal volume of loading buffer (98% fomamide, 0.025% bromophenol blue, 0.05% xylene cyanol) was added to the amplified products, following denaturation at 94^{o}C for 5 min. The products were separated on 6% (w/v) denaturing polyacrylamide gels and visualized with silver staining (Bassam *et al*., 1991)

Results and discussions

Primary callus induction and embryogenic callus induction

After four weeks of culture the young leaf segments in MS-I medium containing 2.5 mg/l dicamba turned to a brown color with malformed shape and neoformation of whitish callus tissues with a round or rod shape (nodule-like structure) occurred. Origin of the callus was mostly from cut surfaces and veins. After two to three times of subculture (4-week intervals, totally 8-12 weeks) primary callus obtained from MS-I to MS-P the callus developed into 3 distinct types. One is fast growing callus. This type of callus was characterized as a fast growing friable creamy callus (FGC). The fresh weight of this callus increased very fast. Doubling time (Dt), time required for increasing fresh weight two times, was 3-4 weeks. The second type was yellow embryonic, so called, embryogenic callus (Fig. 1A). In this callus naked embryoids were found. The third type was yellow compact, so called nodular callus (Fig. 1B). In this type of callus, compact meristemoid structure was observed. Dicamba at 0.1-0.5 mg/l containing MS-P medium promoted the second type of callus whereas concentration at 1.0 mg/l promoted both first and third type of callus (Table 1). Within 10 year of culture, various stages of somatic embryos from globular to haustorium (mature somatic embryo) were obtained. In some cultures, haustorium embryos (HEs) developed from embryogenic callus after 3 weeks of subculture.

Table 1. Effect of concentration dicamba containing MS-P medium on the development of primary callus after culture for 12 weeks

Conc. of dicamba (mg/l)	No. of cultures	Percentage Development of primary callus			
		Fast growing callus		Embryoid forming callus	
		8 weeks	12 weeks	8 weeks	12 weeks
0.1	30	16.67a	61.11a	3.33	3.33a
0.5	24	0	31.57b	0	3.33a
1.0	37	2.70b	7.14c	0	0b
F-test		*	*	*	*
C.V.(%)		16.71	2.57	13.00	7.68

* Significant difference at p = 0.05
Means sharing letter in common within column are not significant difference by DMRT.

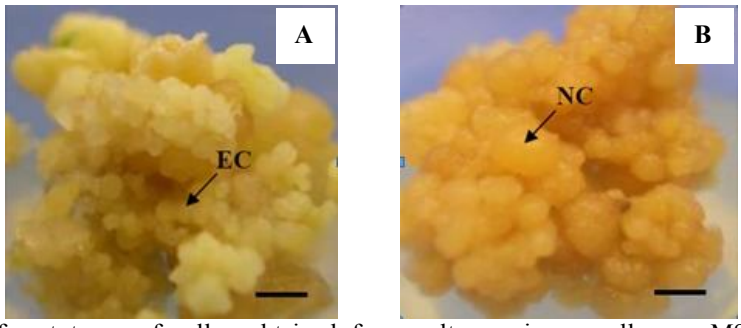

Fig. 1. Different types of callus obtained from culture primary callus on MS-P medium supplemented with 0.1-0.5 mg/l dicamba (A) and 1.0 mg/l dicamba (B)

Induction of secondary somatic embryo (SSE)

SSE is the process of induction of new somatic embryos from pre-existing embryos (Raemakers *et al*., 1995). 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. SSE is a new cycle of somatic embryogenesis which could be induced directly from HE. Basal MS medium supplemented with 0.1 mg/l dicamba in the presence of 0.2M sorbitol resulted in the best SSE induction both percentage (100) and number of SSE (21.55 SSEs/HE) (Table 2) while the other concentrations gave lower response in SSE formation. Even sucrose yielded number of SSEs slightly higher but percentage of SSE formation was far lower. Those SSEs were white, opaque and torpedo in shape which arose directly from peripheral layer of basal part of HE (Promchan and Te-chato 2007). SSEs were also reported to be induced indirectly from SE-derived cotyledon in cotton (Raemakers *et al*., 2000). In terms of chemical factor, not only plant growth regulators play role on SSE formation but sugars and

polyamines also act as promoting substances. However, the key factors for inducing SSE depended upon plant species. For oil palm, 0.1 mg/l dicamba in combination with 0.2 M sorbitol proved to be the optimum for SSE production. Polyamine; spermine, spermidine has been reported to induce SSE from culturing zygotic embryo of oil palm (Rajesh *et al*., 2003), unfortunately, low efficiency of SSE induction obtained. In banana, MS supplemented with 10% coconut water produced rapidly proliferating embryogenic callus that developed into secondary somatic embryos (SSE) (Khalil *et al*., 2002).

Table 2. Effect of concentration of sorbitol on SSE formation after culturing for 12 weeks

Concentrations of sorbitol (M)	**SSE formation**	
	Number	Percentage
0.1	11.55b	21.11c
0.2	21.55a	100a
0.3	2.22c	45.04b
F-test	*	*
C.V.(%)	7.76	4.40

* Significant difference at p = 0.05

Means sharing letter in common within column are not significant difference by DMRT.

Germination of secondary somatic (SSE)

Mature SSE possessed well-defined whitish opaque torpedo-stage morphology with a cylindrical, distinct apical dome with leaf primordia and crown region from which shoot initials and root initials developed (Fig 3A). Accordingly, SEE formed from the basal part of HE could germinate on PGR-free medium at the highest percentage of 78. While SE could germinate at only half (40%) of SSE (Fig. 2). SSE induced on PGR-free MS medium supplemented with 0.2 M sorbitol produced both shoot and root and ready for germination of complete plantlets (Fig. 3B). On this medium, which contained no plant growth regulators, SSE gave rise to small plantlets within 30-45 days (Fig. 3C). The use of SSE could provide an efficient solution to the problems limiting plant regeneration in oil palm like those reports in banana cultivars (Khalil *et al*., 2002). This also suggested that SSEs are probably of unicellular origin, making them an excellent candidate for genetic transformation since the potential for production of chimeric plants is low.

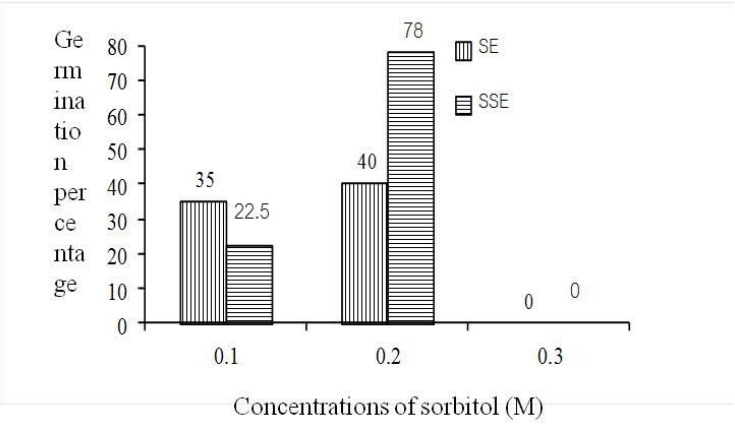

Fig. 2. Effect of concentrations of sorbitol on germination percentage of SE and SSE.

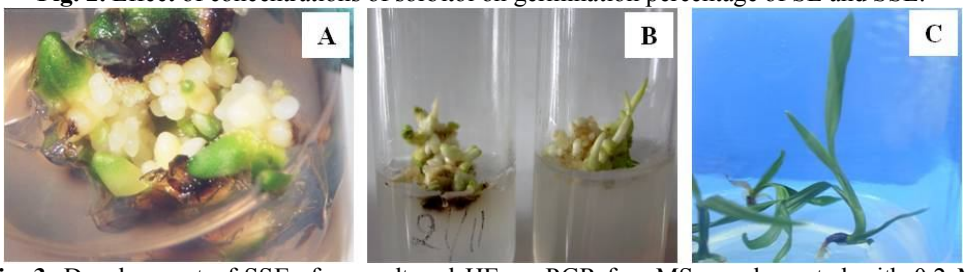

Fig. 3. Development of SSEs from cultured HE on PGR-free MS supplemented with 0.2 M sorbitol for 12 weeks (A, B) subsequent to germination on PGR-free MS without sorbitol after 8 weeks of transfer (C).

Assessment of genetic instability of long-term regenerated plantlets via SSR marker

A high quality of genomic DNA at quantity more than 80 ng could be isolated from first and second leaves of vitro-seedlings (Fig. 4). Amplification of those genomic DNA by SSR technique with 9 primers revealed that primer EgCIR0008 gave a high resolution of DNA profiles which could be used for assessment of genetic instability in long-term regeneration plantlets from SSE.

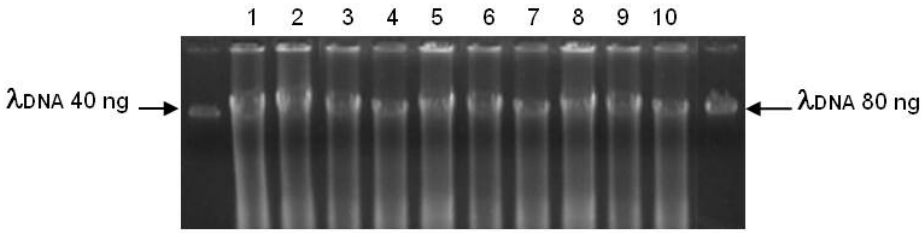

Fig. 4. Qualitative measurement of genomic DNA from first and second leaves of ten vitro-seedlings in comparison with λ DNA.

That primer gave the specific three bands of DNA ranging from 650 to 750 bps with the same pattern among ten stands of vitro-seedlings (Fig. 5). The results obtained from this study suggest that there is no somaclonal variation or genetic instability occur in plantlets regeneration from ten-year-old embryogenic callus of oil palm through SSE formation. 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 (Nayak *et al*., (2003In oil palm, SSR analysis was used to examine genotype identification (Billotte *et al*., (2001, genetic mapping (Billotte *et al*., (2005and population genetic (Singh *et al*., (2007Using SSR technique, various authors have reported the absence of genetic variation in oil palm. Thawaro (2009) reported that SSR analysis of regenerated plantlets with EgCIR1772 primer provided clear DNA patterns and monomorphism band. The result revealed that there was no somaclonal variations detected by this technique. Sanputawong (2010) also reported that SSR marker with primers EgCIR0008 gave a uniformity of plantlets derived from immature zygotic embryo culture. The absence of any sign of somaclonal varation from this study suggests that our secondary embryogenesis system did not induce the changes in gene structure, which had remained stable throughout more than 10 years that all lines had been maintained *in vitro*.

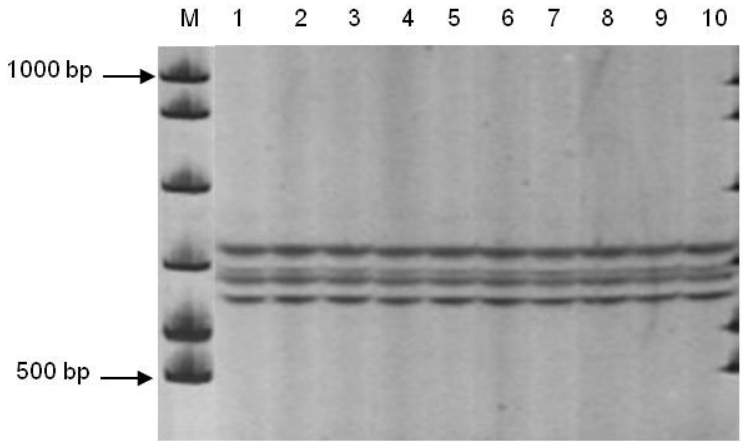

Fig. 5. SSR patterns from young leaves of ten vitro-plantlets of oil palm with primer EgClR0008. Those plants were obtained through SSE formation from embryogenic callus which was maintained on MS-P solidified medium for more than 10 years. (M: standard DNA)

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