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Application of molecular markers in the hybrid verification and assessment of somaclonal variation from oil palm propagated in vitro

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ABSTRACT: Randomly amplified polymorphic DNA (RAPD) and simple sequence repeat (SSR) were used to assess the hybrid verification and somaclonal variation of the cross 366 (D) × 72 (P) of *Elaeis quineensis* Jacq. DxP. DNA from mature zygotic embryo (MZE) was isolated and detected by seven primers of RAPD and eight primers of SSR. Half MZEs consisting of coleoptiles regions were cultured on Murashige and Skoog (MS) medium plus various kinds and concentrations of auxins. Variation of somaclone obtained through somatic embryogenesis was detected at the proliferation stage of the culture period. The result revealed that all random primers and the SSR primers tested could amplify parental DNA. Primer OPT06 (for RAPD) and EgCIR1772 (for SSR) provided clear DNA patterns and could be used for verifying the hybridity of the cross 366 (D) × 72 (P). The highest frequency of nodular callus formation at 87.5% was obtained on MS medium supplemented with 2.50 mg/l of 3,6-dichloro-o-anisic acid (dicamba). This was significantly different from other kinds and concentrations of auxins. Somatic embryo line at globular stage derived from each half MZE showed no variation in RAPD banding patterns with primer OPT06. It was concluded that no somaclonal variation occurred in our propagation system by RAPD marker, no major genetic changes were observed, and this augurs well for the propagation system being employed since somaclonal variation may be minimized or absent altogether.

KEYWORDS: half-mature zygotic embryo

INTRODUCTION

Oil palm (*Elaeis guineensis* Jacq.) is a diploid monocotyledon belonging to the family Arecaceae. It is an economically important source of the most traded vegetable oil in the international market, and is increasingly used in the food industry[1]. In Thailand, the government aims to increase palm oil production in order to serve the biodiesel industry due to fluctuations 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 domestic consumption of biodiesel will grow to 31.3 billion litres per year in the next six years. By the year 2012, it is estimated that the area needed to cultivate oil palms will be 1.44×10^6 km^2.

All modern commercial palms are F1 hybrids between selections with small or no kernels (pisifera) and large thick kernels (dura), but the hybrids (tenera) show very high variation in oil yield with the best plants yielding 40% more than average. As oil palm is normally propagated by seed, there is a high variation in the field. Also, the available sustainable genetic trait production through traditional breeding is limited. Hence low palm oil yield is the norm. A shortage of good hybrid oil palms is the major problem of oil palm cultivation in Thailand. To optimize time and costs, 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. In order to overcome these limitations, plant micropropagation is used for plant breeding, and clonal propagation of oil palm through tissue culture is common[2,3]. There have been previous reports of oil palm micropropagation through somatic embryogenesis[4–7] and embryogenic suspension[8]. However, most of the callus cultures were successful when induced on a high concentration of 2,4-D containing medium[9,10]. The use of a high concentration of 2,4-D has been implicated as a cause of somaclonal variation in different species of plant including oil palm[11,12]. In order to reduce the potential for tissue culture-induced variation, protocols based on the use of lower levels of auxins should be considered. Molecular markers have been used in many agricultural areas, especially plant breeding, and

biochemical markers such as isozyme analysis are an established method that can be used for determination of hybridity. However, the use of this method is limited due to many factors affecting protein expression including the developmental stage of plant tissue and the environment. A low number of markers restricting polymorphism may also affect the utility of these markers [13].

Randomly amplified polymorphic DNA (RAPD) is a valuable tool for identifying genetic variation because it is inexpensive, quick, and simple [14]. It permits the identification of DNA polymorphisms and can be used to amplify particular fragments of genomic DNA [15]. DNA profiles have been electrophoresed to analyse the genetic relationships of plant species [16]. RAPD analysis is based on the presence or absence of polymorphisms in individuals or groups of individuals [17]. In *Elaeis*, a RAPD marker has been employed for the analysis of genetic variation among African germplasm accessions [18] and was also used to examine the possibility of detecting somaclonal variation in clonal oil palm [19].

Simple sequence repeat (SSR) markers provide one of the best PCR marker systems and the importance of SSR as a source of markers for plant genetics have been confirmed. With regards to oil palm, it was demonstrated that simple repetitive DNA was present in abundance in the oil palm [20]. It can be used to screen oil palm genomic DNA for di-, tri-, tetra- and pentanucleotide repeats and found them to be widely distributed in the oil palm genome. Initial attempts to construct SSR enriched genomic libraries were not very successful, as less than 1% of the clones appeared to contain SSR motifs [21]. Later, other researchers [22] reported the successful construction of an oil palm SSR enriched genomic library, with 72% of the clones containing SSR motifs. They went on to demonstrate the applicability of the SSR markers in revealing the genetic relationships of populations of the genus *Elaeis* in accordance with their known geographical origins. They also demonstrated that the markers developed for one species, *E. guineensis*, were applicable in a second species, *E. oleifera*. Hence both micropropagation and molecular marker assisted selection is of great importance in the propagation of oil palm on the commercial scale. Before reaching that point, hybridity of the mature zygotic embryo (MZE) should be verified at an early stage subsequent to mass propagation through verified MZE. In the present paper, we describe verification of hybrid cross using half seeds through RAPD and SSR markers using MZE halves and multiplying the remaining MZEs through somatic embryogenesis.

MATERIALS AND METHODS

Plant material

MZEs of 'Tenera' hybrid, derived from the cross 366 (D) $\times$ 72 (P) 180 days after pollination were excised by the following protocol. The mesocarp was 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 5 mm $\times$ 5 mm $\times$ 8 mm and used as explants for culture. The cubes were surface sterilized by 70% alcohol for 1 min and 20% Clorox (containing 0.5 ml of Tween-20 emulsifier per 100 ml solution) for 20 min, followed by three washings with sterile distilled water in a laminar flow station. The embryos were aseptically removed from the kernel and cultured on culture medium.

Hybrid verification via RAPD analysis

MZEs were cultured on Murashige and Skoog (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. The first half of MZE consisting of mainly haustorium were collected (15–20 mg) and brought to DNA isolation according to the technique described in Ref. 23. 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. The RAPD analysis was performed according to the method of Saichon [24]. Each amplification mixture of 25 µl contained 2.5 mM $MgCl_2$, 10× *Taq* buffer, 100 µM of each dNTP, 0.3 mM of each 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 0.5× TBE buffer at 100 V. The gels were stained with ethidium bromide for 15 min and 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 medium 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 were cut

in half. The first halves consisting mainly of haustorium were collected (15–20 mg) and used to isolate DNA. SSR analysis of genomic DNA was carried out using 9 microsatellite loci amplified in oil palm using 9 primers (EgCIR008, EgCIR0243, EgCIR0337, EgCIR0409, EgCIR0446, EgCIR0465, EgCIR0781, EgCIR0905, and EgCIR1772) as described in Ref. 22. Amplification of genomic DNA[25] was done in a 10 µl mixture containing 2.5 mM $MgCl_2$, 10× *Taq* buffer, 100 µM of each dNTP, 0.3 mM of each 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, 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 and visualized with silver staining[26].

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

The remaining halves of the MZEs consisting of shoot apical was inoculated in culture tubes containing 10–15 ml of modified 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 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 autoclaved at 1.05 kg/cm^2, 121 °C for 15 min. The cultures were placed under light conditions (3000 lux illumination for 16 h photoperiod) at 25 ± 2 °C and subcultured every 4 weeks on the same medium component for 3 months. The percentage of cultures that produced callus, the type of callus, and the number of the embryos per tube were recorded after 1 month for 3 months of culture by counting under a stereo microscope (Nikon, SMZU).

Proliferation of embryogenic callus and formation of somatic embryo

Nodular calluses (approximately 5 nodule/explant) were carefully separated and inoculated on MS medium supplemented with 1 mg/l dicamba, 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 was recorded for 3 months.

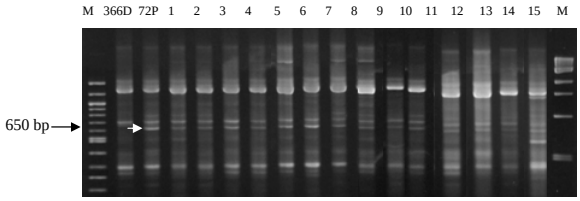

Fig. 1 RAPD patterns in hybrids and parents of the cross 366 (D) × 72 (P) obtained with primers OPT06. In this and the next figure, the amplification products were compared on the basis of molecular size. Lane M: standard DNA (100 bp plus DNA ladder). Lanes P and D: fragments from parents. Lanes 1–15: fragments from hybrids.

Assessment of somaclonal variation by RAPD analysis

DNA samples of somatic embryo at globular stage from of the proliferation phase for 3 months of culture were amplified with OPT06 primer. The RAPD analysis was performed under the same conditions as described earlier. PCR products were then electrophoresed in 1.5% (w/v) agarose gels in 0.5× 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.

Statistical analysis

For experimental design and statistical analysis, completely randomized design with 4 replicates (each with 10 embryos) was performed. Data was analysed using ANOVA.

RESULTS

Hybrid verification via RAPD analysis

All primers could amplify and provided polymorphic patterns of parents. The number of bands for each primer varied from 8 to 17 with an average of 14 fragments per primer. The size of the amplified products ranged from 100 to 1517 bp. A total of 97 RAPD fragments were scored from the seven random primers. The results revealed that there was only one primer, OPT06, that provided a clear DNA pattern. Results of DNA pattern of the cross 366 (D) × 72 (P) had specific fragment (650 bp) and which could be used to distinguish hybrid between dura and pisifera. All hybrids showed the DNA patterns between the two parents and more additive bands (Fig. 1).

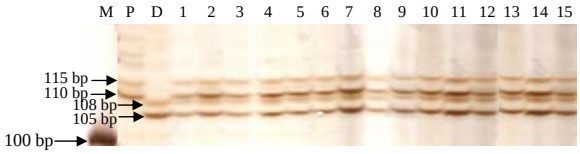

Fig. 2 SSR patterns in hybrids and parents of the cross 366 (D) × 72 (P) obtained with primers EgCIR1772.

Hybrid verification via SSR analysis

All primers could be amplified and provided polymorphic patterns of DNA from the parents. The number of bands for each primer varied from 2 to 6 with an average of 2 fragments per primer. The size of the amplified DNA ranged from 100 to 250 bp. Among those primers mEgCIR1772 provided a clear DNA pattern. This primer showed the greatest capacity for distinguishing polymorphic fragments in half-embryo cultured from the parent 366 (Dura) and 72 (Pisifera). The results from the DNA pattern of the cross 366 (D) × 72 (P) showed a specific fragment and could be used to distinguish between dura and pisifera palms. All hybrids showed the DNA patterns between the two parents and more additive bands according to SSR analysis. One hundred samples were heterozygous with primer mEgCIR1772 showing both a male parent-specific band at 115 and 110 bp, and a female parent-specific band at 108 and 105 bp (Fig. 2).

Effect of various kinds and concentrations of auxins on the type of callus formation

Half MZEs consisting of coleoptile of the cross 366 (D) × 72 (P) swell after 10–14 days of culture and started to form callus after 4–5 weeks of culture. Most of the auxins promoted callus formation from half MZEs of the cross 366 (D) × 72 (P) after 6 weeks of subculture. Four types of calluses could be distinguished from the cultures. Friable calluses (FCs) were yellow, translucent and succulent. Compact calluses (CCs) were muddy white and compact. Nodular calluses (NCs) were yellow or pale yellow and compact. Root-like calluses (RLCs) were white, soft and elongate in shape. Kinds and concentrations of auxins supplemented in culture medium had a significant effect on the type of calluses. Characteristics of the callus obtained in NAA, 2,4-D and dicamba containing the medium were quite different. Dicamba provided a yellow compact callus (the nodular callus) whereas 2,4-D gave both a white friable callus and a white elongative soft callus. NAA could not cause callus formation (Fig. 3). The highest percentage of nodular callus (87.5%) was obtained from 2.50 mg/l dicamba after 3 months of culture (Table 1).

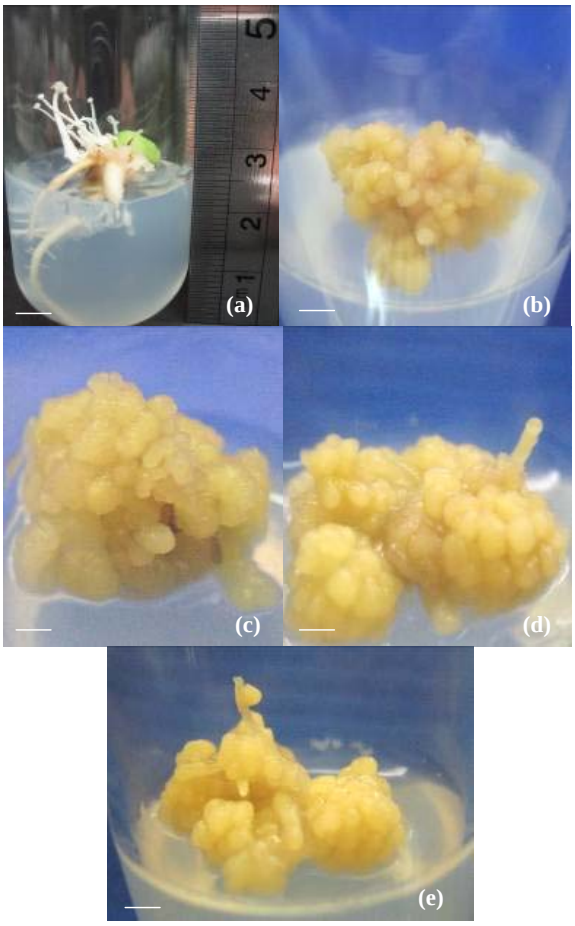

Fig. 3 Callus formation from culturing mature zygotic embryo cultured on solid MS supplemented with various hormones for 3 months. Callus cultured on MS medium supplemented with (a) 40 mg/l NAA, (b) 2.5 mg/l 2,4-D, (c) 5.0 mg/l 2,4-D, (d) 2.5 mg/l dicamba, (e) 5.0 mg/l dicamba (scale bar: 2.5 mm).

Proliferation of embryogenic callus and formation of somatic embryo

Approximately 5 nodules of NC from the cross 366 (D) × 72 (P) were gradually increased by increasing the time of culture. After 3 months of culture, MS medium supplemented with 1 mg/l dicamba gave embryogenic callus proliferation (Fig. 4a). Somatic embryo (SE) formation was obtained from the same medium components after 2 months of proliferation phase (data not shown, Fig. 4b). The result of our experiments showed that the culture period played a significant role on the percentage of somatic embryo formation. Three months of culture gave the best

Table 1 Effect of various kinds and concentrations of auxin on type of callus formation from mature zygotic embryos of the cross 366 (D) × 72 (P) after culture for 3 months.

Auxins	Concentrations (mg/l)	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	40.00	15.00^{a}	0.00^{b}	2.50^{c}	27.50^{bc}	3.54 ± 1.90^{b}	2.36 ± 1.50
2,4-D	2.50	2.50^{b}	2.50^{b}	37.50^{b}	12.50^{c}	4.27 ± 1.09^{b}	1.60 ± 0.55
	5.00	20.00^{a}	12.50^{a}	70.00^{ab}	52.50^{ab}	6.46 ± 6.19^{b}	2.90 ± 1.54
Dicamba	2.50	17.50^{a}	15.00^{a}	87.50^{a}	70.00^{a}	18.78 ± 17.49^{a}	5.22 ± 4.60
	5.00	22.50^{a}	12.50^{a}	45.00^{b}	22.50^{bc}	11.57 ± 9.47^{ab}	4.03 ± 2.38
C.V. (%)		30.05	34.80	33.82	24.50	37.17	38.98

Means followed by the same letter within a column do not different significantly ($p < 0.05$). C.V. = Coefficient variance

Table 2 Effect of time period on somatic embryo formation of the cross 366 (D) × 72 (P) after culture 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 embryo / explant	
	Globular stage	Haustorium stage	Globular ± SD	Haustorium ± SD
1	4.63^{c}	1.57^{c}	1.78 ± 0.51^{c}	0.23 ± 0.05^{b}
2	10.22^{b}	3.44^{b}	6.11 ± 2.94^{b}	1.38 ± 0.78^{b}
3	17.32^{a}	7.92^{a}	13.38 ± 3.65^{a}	4.00 ± 1.33^{a}
Mean	10.72	4.31	7.09 ± 2.36	1.87 ± 0.72
C.V. (%)	14.34	12.92	11.48	27.40

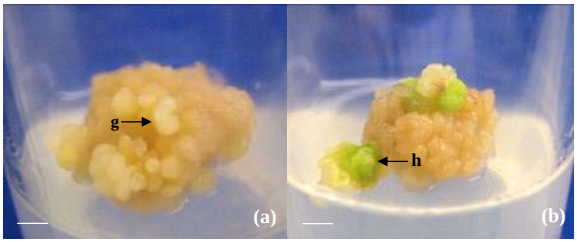

Fig. 4 Somatic embryo formation. (a) Globular stage (G) and (b) haustorium stage (H) after culturing NC for 3 months (bar = 2.5 mm).

results. (Table 2).

Assessment of somaclonal variation by RAPD analysis

The variation of the somatic embryos at the globular stage from the cross 366 (D) × 72 (P) were assessed using RAPD. Primers used in this technique were shown to amplify the products of DNA (Fig. 5). Among 15 somatic embryos there was no variation detected in the DNA profile. We concluded that no somaclonal variation occurred in our propagation system by RAPD marker, and no major genetic changes were observed. This augurs well for the propagation system being of use since somaclonal variation may be minimized or absent altogether.

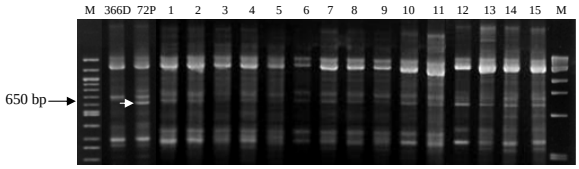

Fig. 5 RAPD pattern of somatic embryo line derived from MZE obtained with primers OPT06. The amplification products were compared on the basis of molecular size. Lane M: standard DNA (100 bp plus DNA ladder). Lane P and D: profile of DNA fragments from parents. Lane 1–15: profile of DNA fragments from hybrids.

DISCUSSION

Different markers have been used in many agriculture areas, especially plant tissue culture. In the present study, two DNA marker systems, RAPD and SSR were used for oil palm F1 hybrid evaluation. The result of our experiments indicated that RAPD markers can be used for identification of hybridity. The application of OPT06 as a primer could amplify hybrid DNA and can be used for testing hybridity of the cross 366 (D) × 72 (P). These results are similar to those reported in chestnut[27]. They indicated that RAPD markers were successfully used 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 the complex heredity background of each plant heterosis[28–30]. 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 primers. Liu et al[31] also reported that primers NAURP 403 and 1123 produced female parent-specific markers 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 from the female and male parent-specific markers in some marker loci. This is due to hybrid vigour having a positive relation with genetics[32]. Similarly, residual amounts of heterozygosity can also result from the female and male parent-specific markers in oil palm. Moreover, there are still some other bands of DNA aside from the parent-specific markers indicating hybrid vigour. Xiao et al[32] also showed that hybrid vigour has a positive relation with extra banding of DNA. The result from our study suggests that heterosis is positively correlated with the extra DNA banding that appeared in the DNA profile of F1 and the accumulation of all the parental genes in the F1 reveals hybrid vigour. The heterosis observed in the F1 hybrid could be combined alleles associated with hybrid vigour[33]. This could be minor bands that arise out of using RAPD primers in combination with low annealing temperature in the PCR reaction. This combination as reported in many early RAPD experiments can give rise to unspecific binding of the primers to many regions some of which can amplify as minor bands in some samples but are absent in others. Since RAPD theoretically amplifies all regions of the genome, at least some of the products will be uniparentally inherited organelle sequences, as highlighted by Thormann et al[34] in Brassicaceae and by Lorenz et al[35] in Chenopodiacea. The RAPD marker sensitivity to the conditions under which the analysis is conducted may create problems with reproducing experiments under conditions of another laboratory[36]. 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. For SSR markers, the SSR pattern among the cross 366 (D) $\times$ 72 (P) with primer mEgCIR1772 showed the presence of both male and female fragments of DNA. These results were similar to those reporting the use of SSR markers for testing seed genetic purity of commercial hybrid tomato cultivars[37]. Our results show that SSR markers are more effective for verification and identification of hybridity of oil palm than RAPD markers.

For culturing of MZE or half MZE, MS medium supplemented with 2.50 mg/l dicamba gave the highest NC formation at 87.50%, which was significantly different from the other auxins. Kinds and concentrations of auxins supplemented in culture medium had a significant effect on the type of calluses. Characteristics of the callus obtained in NAA, 2,4-D and dicamba containing the medium were quite different. The highest percentage of nodular callus was obtained from 2.50 mg/l dicamb. Dicamba was effective in inducing callus from embryo culturing of wheat (*Triticum aestivum* L.)[38]. A similar result was also found in immature embryo cultures of winter wheat[39] and spring wheat cultivars[40]. Dicamba is a promising auxin which has been reported to be effective in promoting direct and indirect embryogenic callus induction from cultured mature zygotic embryo and young of leaf oil palm[41]. The time consumed for callus induction in culture medium supplemented with dicamba was shorter than with 2,4-D and NAA. A similar result was obtained from culturing young leaves of the same plant[42,43]. In addition, medium containing dicamba was reported to induce nodular structure from both epidermal cells and vascular tissues while 2,4-D induced this only from the epidermis[44].

For proliferation of embryogenic callus and formation of SE, MS medium supplemented with 1 mg/l dicamba gave proliferation of embryogenic callus and formation of SE. Dicamba was found to be the best auxin for mass propagation in vitro of both seedling and mature oil palm[45]. In addition, embryoids developed on medium containing 0.1 mg/l dicamba were found to be superior in inducing early stages of embryoid subsequent to further development of mature or haustorium embryoids[46]. Some authors reported that a low concentration of dicamba promoted somatic embryogenesis from immature inflorescence[47]. A similar result was obtained in callus culture of *Areca catechu*. A decrease in the concentration of dicamba increased the proliferation rate of callus and also the development of somatic embryos[48]. From the present study, it is clear that somaclones obtained from our protocol were uniform according to the RAPD markers. Using RAPD, various authors have reported the absence of genetic variation in trees like *Picea mariana*[49], *Festuca pratensis*[50], and *Pinus thunburghii*[51]. Our results suggest that RAPD 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 transfer of hardened plants to field.

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