ภาคผนวก

ตารางภาคผนวกที่ 1 แสดงปริมาณดีเอ็นเอ (pg) ของคัพภะปาล์มน้ำมันที่สกัดด้วยสารละลายนิวเคลียส 5 ชนิด โดยมีพืชอ้างอิงมาตรฐานคือถั่วเหลือง

Nuclear lysis buffers	Embryos (pg)			Average (pg)
	Rep1	Rep2	Rep3	
LB01	**3.82**	**3.74**	**3.70**	**3.75**
WPB	2.98	2.73	3.01	2.91
Otto's	4.23	5.22	5.21	4.89
Tris.$MgCl_2$	2.84	2.76	2.80	2.80
Galbraith	3.07	1.89	1.79	2.25

ตารางภาคผนวกที่ 2 แสดงปริมาณดีเอ็นเอ (pg) ของใบอ่อนปาล์มน้ำมันอายุ 1 เดือน ที่สกัดด้วยสารละลายนิวเคลียส 5 ชนิด โดยมีพืชอ้างอิงมาตรฐานคือถั่วเหลือง

Nuclear lysis buffers	Leaf positions (1 month-old seedling) (pg)			Average (pg)
	Leaf apices	Leaf middles	Leaf bases	
LB01	2.90	1.09	1.86	1.95
WPB	4.68	4.53	**3.69**	4.30
Otto's	1.11	2.33	2.81	2.08
Tris.$MgCl_2$	-*	1.23	1.54	1.39
Galbraith	1.73	1.16	0.78	1.22

*ไม่สามารถสกัดนิวเคลียสออกมาได้

ตารางภาคผนวกที่ 3 แสดงปริมาณดีเอ็นเอ (pg) ของใบอ่อนปาล์มน้ำมันอายุ 3 เดือน ที่สกัดด้วยสารละลายนิวเคลียส 5 ชนิด โดยมีพืชอ้างอิงมาตรฐานคือถั่วเหลือง

Nuclear lysis buffers	Leaf positions (3 month-old seedling) (pg)			Average (pg)
	Leaf apices	Leaf middles	Leaf bases	
LB01	1.51	2.17	3.64	2.44
WPB	3.50	4.02	3.93	**3.82**
Otto's	-*	-*	-*	-
Tris.$MgCl_2$	0.72	0.70	0.83	0.75
Galbraith	-*	-*	0.55	0.55

*ไม่สามารถสกัดนิวเคลียสออกมาได้

ตารางภาคผนวกที่ 4 แสดงปริมาณดีเอ็นเอ (pg) ของใบอ่อนปาล์มน้ำมันอายุ 6 เดือน ที่สกัดด้วยสารละลายนิวเคลียส 5 ชนิด โดยมีพืชอ้างอิงมาตรฐานคือถั่วเหลือง

Nuclear lysis buffers	Leaf positions (6 month-old seedling) (pg)			Average (pg)
	Leaf apices	Leaf middles	Leaf bases	
LB01	2.43	2.82	3.03	2.76
WPB	4.90	5.48	4.36	4.91
Otto's	2.1	2.46	4.75	3.10
Tris.$MgCl_2$	-*	-*	3.46	3.46
Galbraith	2.09	1.95	1.78	1.94

*ไม่สามารถสกัดนิวเคลียสออกมาได้

ตารางภาคผนวกที่ 5 แสดงปริมาณดีเอ็นเอ (pg) ของใบอ่อนปาล์มน้ำมันอายุ 12 เดือน ที่สกัดด้วยสารละลายนิวเคลียส 5 ชนิด โดยมีพืชอ้างอิงมาตรฐานคือถั่วเหลือง

Nuclear lysis buffers	Leaf positions (12 month-old seedling) (pg)			Average (pg)
	Leaf apices	Leaf middles	Leaf bases	
LB01	1.47	2.12	2.47	2.02
WPB	4.27	3.58	3.61	**3.82**
Otto's	3.08	2.64	3.24	2.99
Tris.$MgCl_2$	2.51	2.52	3.09	2.71
Galbraith	1.60	1.54	-*	1.57

*ไม่สามารถสกัดนิวเคลียสออกมาได้

ตารางภาคผนวกที่ 6 แสดงปริมาณดีเอ็นเอ (pg) ของใบอ่อนปาล์มน้ำมันรวมทุกระยะการเจริญเปรียบเทียบกับคัพภะปาล์มน้ำมัน ที่สกัดด้วยสารละลายนิวเคลียส 5 ชนิด โดยมีพืชอ้างอิงมาตรฐานคือถั่วเหลือง

Nuclear lysis buffers	Average all stages of oil palm seedling (pg)			Embryos (pg)
	Leaf apices	Leaf middles	Leaf bases	
LB01	2.08	2.05	2.75	**3.75**
WPB	4.34	4.40	**3.90**	2.91
Otto's	2.10	2.48	3.60	4.89
Tris.$MgCl_2$	1.62	1.48	2.23	2.80
Galbraith	1.81	1.55	1.04	2.25

Manuscript

Cultivars classification approach in oil palm by using flow cytometry

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Abstract

Efficiencies in each nuclear lysis buffer on flow cytometric analysis (FCM) of oil palm were compared for cultivars classification. Five nuclear lysis buffers (LB01, WPB, Otto's, Tris.$MgCl_2$ and Galbraith) were used to prepare samples from embryos and unopened leaves of one to twelve months-old seedlings. Although these buffers showed distinct peaks of PI fluorescence for oil palm embryos, the buffers differed considerably in Fluorescence intensity (FL), Coefficient of variance (%CV), Debris factor (%DF) and Yield factor (YF) values when analyzed with their seedlings. The results showed that LB01 was the most suitable buffer (lowest %CV and high FL value) for oil palm embryos resulting in 3.7 pg of 2C DNA. For all seedlings, only WPB gave the highest value of FL and lowest value of %CV resulting approximately in 3.8 pg of 2C DNA whereas the highest value of YF and lowest value of %DF were mostly found in LB01. By the way, only WPB showed consistency position of PI fluorescence histograms when analyzed with all seedlings. WPB was therefore used to classify the cultivars of hybrid Tenera and its parents (Dura and Pisifera) by means of DNA contents. DNA contents of Dura and Pisifera were ranged from 6.3 - 7.6 and 5.3 – 6.1 pg, their genome size (1C DNA contents) is therefore greater than 3000 and 2000 Mbp, respectively. The results confirmed that Dura, Pisifera and Tenera cultivars could be classified by using FCM-derived DNA contents.

Keywords: 2C DNA value, cultivars, flow cytometry, nuclear lysis buffers, oil palm

Introduction

Information of ploidy levels and DNA content is important in any breeding program of plant varieties. Based on fruit structure, oil palm has been systematically classified into 1. Dura (thick shell; less mesocarp) 2. Pisifera (shelless; embryo rarely formed) and 3. Tenera, the Dura×Pisifera hybrid (thin shell; more mesocarp: 60-95%), with high oil content. Tenera is a valuable economically important source of vegetable oil and is increasingly used to power vehicle namely biodiesel. Since there is high potential of conventional cross breeding among Dura and Pisifera cultivars in the first filial hybrid trait, DNA content of the hybrid is probably variable. However, intra-specific identification of these species remains difficulties, since they have very similar morphological features at vegetative phase. The only possible way to identify them is the presence or absence of endocarp or shell of the fruits (Sathish and Mohankumar, 2007). Investigating the variability within the species is therefore important to determine the oil yield representation and to identify unique fruit types that would help in developing improved cultivars (Narasimhamoorthy *et. al*, 2008). Although there are many potential DNA markers to investigate cultivated clone of oil palm (Mayes *et al.*, 2000; Barcelos *et al.*, 2002; Zehdi *et al.*, 2004), difficulties have been encountered when using these procedures on a large scale (Rival *et al.*, 1997).

Flow cytometry (FCM) is the method offering a simple, rapid, accurate and convenient analysis for determining ploidy levels of DNA, assessment and analysis of the cell cycle of large cell populations (Winkelmann *et al.*, 1998; Dolezel, 1991). The extension of FCM analysis was recently supported by the relative complexity of nuclear lysis buffer compositions, which involves peak position and quality and values of DNA content in various species. A systematic comparison of nuclear lysis buffers was done by Loureiro *et al.* (2006a,b) and Loureiro *et al.* (2007) who compared six of the most common buffers differing in chemical composition: Galbraith, LB01, Otto's, Tris.$MgCl_2$, GPB (General Purpose Buffer) and WPB (Woody Plant Buffer) (Loureiro *et al.*, 2007) buffers. The buffers have been considered by giving the parameters including higher FL and YF, and lower %CV and DF (Greihuber *et al.*, 2007). However, given the different chemical composition and distinction of plant tissues promoting performed differently in each buffer (Loureiro *et al.*, 2006b). Therefore it is unacceptable to use only single buffer with every plant species (Dolezel and Bartos, 2005).

There are only three previous FCM analyses of oil palm reported by Rival *et al.* (1997), Srisawat *et al.* (2005) and Madon *et al.* (2008). The results of their DNA content have been resembled for 3.7 pg (Rival *et al.* 1997; Srisawat *et al.* 2005) and slightly differed to 3.8 pg (Madon *et al.*, 2008). Not only the differences of the techniques and types of standard used effecting on discrepancies of oil palm DNA content, type of nuclear lysis buffers is also revealing (Loureiro *et al.*, 2006b; Loureiro *et al.*, 2007). Therefore an appropriate nuclear lysis buffer for oil palm FCM analysis should be investigated more extensively for any further breeding and production programs of oil palm. The chosen buffer used to classify oil palm cultivars was usually characterized by giving higher FL and YF and lower %CV and DF (Loureiro *et al.*, 2007).

Our attentions focus on the discrimination of the effective nuclear lysis buffers from FCM analysis of oil palm embryos and seedlings replicating into three unopened-leaf positions (leaf-apices, leaf-middles and leaf-bases) and establishment the effective marker for classification of oil palm cultivars by using DNA content values.

Methodology

Plant materials

Breaking dormancy-derived seeds, one to twelve months-old seedlings of oil palm (*Elaeis guineensis* Jacq.) cv. Tenera and young leaves of more than ten years-old tree of Deli Dura: (D109, D067, D064, D069 and D068), Pisifera: (Calabar; P109, LA ME; P106, DAMI; P116, Nigeria; P110 and EKONA; P105) and hybrid Tenera (Suratthani1, 2, 3 and 5) cultivars were prepared by the Suratthani Oil Palm Research Center, Suratthani province, Thailand. Seeds of soybean (*Glycine max* cv. Polanka), reference plant, were kindly provided by Dr Jaroslav Dolezel (Institute of Experimental Botany, Olomouc, Czech Republic).

Comparison of five Flow cytometric nuclear lysis buffers

The flow cytometer was a FACSCalibur (Becton Dickinson Biosciences, San Jose, CA) working with CellQuest software (Becton Dickinson) equipped with a 488 nm argon iron laser. Propidium iodide (PI) was measured at 585 nm to read 2C nuclei histograms of 5,000 nuclei per sample. During analysis, after every three samples, the reference plant was used as a controller to check the calibration of the flow cytometer in each buffer by adjusting the gain of *Glycine* to channel 200. All experiments were carried out with 3 replicates per treatment.

Soybean was used as external reference plants (2C=2.50 pg; Dolezel *et al.*, 1994). Unopened leaves (replicating to leaf-apices, leaf-middles and leaf-bases) of one to twelve months-old seedlings and embryos of oil palm and the 2nd - 3rd leaves from the shoot apex of reference plants, approximately 50 mg, were finely chopped with a razor blade in 1.0 ml Tris-$MgCl_2$, WPB, LB01, Galbraith and Otto's extraction buffers containing: [200 mM Tris, 4 mM $MgCl_2$, 0.5%(w/v) Triton X-100 and

3.0%(w/v) polyvinylpyrrolidone (PVP)], [200 mM Tris.HCl, 4mM $MgCl_2$, 2mM Na_2EDTA, 86 mM NaCl, 10 mM sodium metabisulfite, 1% PVP-10, 1%(v/v) Triton X-100, pH 7.5], [15 mM Tris, 2mM Na_2EDTA, 0.5 mM spermine.4HCl, 80 mM KCl, 20 mM NaCl, 0.1% (v/v) Triton X-100, pH 8.0], [45 mM $MgCl_2$, 30 mM sodium citrate, 20 mM MOPS, 0.1% (v/v) Triton X-100, pH 7.0] and [(Otto I: 100 mM citric acid, 0.5% (v/v) Tween 20 pH 2-3), (Otto II: 400 mM Na_2HPO_4, pH 8-9)], respectively. After extraction, 50 microlitres of RNase and PI were added immediately prior to filtering through a 42 micrometre nylon mesh (Pfosser *et al.*, 1995). FCM parameters and DNA contents were recorded and analyzed for selecting the chosen buffer and plant material for classifying oil palm cultivars: FS, SS, FL, %CV, %DF and YF.

FS=Forward scatter as a rough measure of particle's size

SS=Side scatter as a measure of particles optical complexity

FL=Fluorescence intensity of PI-stained nuclei

%CV=G0/G1 peaks as a measure of nuclear intensity and variation in DNA staining.

%DF=Debris background factor as a measure of sample quality

Use the equation:

$$\%DF = \frac{total\ number\ of\ particles - total\ number\ of\ intact\ nuclei}{total\ number\ of\ particles} \times 100$$

YF=Nuclear yield factor in order to compare the quantity of nuclei in suspension.

Use the equation:

$$YF = \frac{\frac{total\ number\ of\ intact\ nuclei}{number of\ second\ run\ (s)}}{weight\ of\ tissue\ (mg)}$$

Estimation of DNA contents for cultivars classification

Young leaves of Deli Dura, Pisifera and hybrid Tenera trees were prepared followed the procedures mentioned above. FCM analysis was carried out by using the chosen buffer and subsequently determined DNA content in each cultivar.

Use the equation:

$$2C\,DNA\ content = \frac{Sample\ G1\ mean\ FL}{Reference\ standard\ G1\ mean\ FL} \times DNA\ content\ of\ reference\ standard$$

Laboratory trial design and data analysis

The experiment was designed in accordance with the Completely Randomized Design (CRD) with two factors, namely: five types of lysis buffer and five growth stages of oil palm seedlings with three replicates by means of three positions of unopened leaves.

The fluorescence histograms were resolved into G0/G1 (2C), S and G2/M (4C) cell-cycle compartments with a peak-reflect algorithm using two Gaussian curves (WinMDI version 2.9). The FCM parameters and DNA contents of oil palm were statistically analyzed by ANOVA (Analysis of Variance) and the significant differences between the contents in each parameter and DNA value were tested against the *F*-distribution at $P<0.05$. Tukey testing was performed for routine multiple mean comparison.

Results

Comparison of five flow cytometric nuclear lysis buffers for parameters estimation

Testing the five nuclear lysis buffers with embryos and one to twelve months-old seedlings of oil palm revealed significant distinctions in all parameters. The use of each buffer resulted in acceptable parameters with oil palm embryos. As seen in Figure 1, the PI-fluorescence peak histograms of oil palm embryos analyzed with all buffers gave a good reading in peak qualities and positions, indicating that each buffer can be used to investigate 2C DNA contents of oil palm embryos which releasing of their nuclei, considerably. Table 1 lists the average of FS, SS, FL, %CV, %DF and YF values, discriminating of suitable buffer for FCM analysis of oil palm by deciding the following criteria: first and second highest of FL and YF and first and second lowest of %CV and DF values.

Nuclei isolated from embryos and seedlings of oil palm with all five buffers had no significant differences in FS and SS values. Interestingly, both of these values revealed their event out of scale on FS and SS logarithm density plots, corresponding too high values of FS and SS (Table1). In most of leaf tissues, an effect similar to the "the tannic acid effect" was observed which involved the occurrence of two populations, higher %CV and DF (Loureiro *et al.*, 2006a). Correspondingly, two populations of particles on cytograms of FS *vs*. SS, high values of %CV and DF were found in this study. Leaf tissues treated with all buffers maintained a high level of FS, SS. %CV and %DF which might effect from "the tannic acid effect" reaction.

The LB01 gave the second highest of FL (299.73) and first lowest of %CV of DNA peak (4.57%) of oil palm embryos, unfortunately the YF value showed unexpected yields in the lowest (0.68 nuclei s^{-1} mg^{-1}). Otto's buffer yielded acceptable histograms with the first highest of FL value for 385.55 and second lowest of %CV

(4.93%) whereas the first lowest of debris factor (98.90%) and second highest YF value (0.96 nuclei s^{-1} mg^{-1}) were found when using Galbraith. The highest value of yield factor was found to be 0.99 nuclei s^{-1} mg^{-1} for WPB buffer. Although, no significant difference among the buffers was obtained in FS, SS, %DF and YF values, there are different significantly in FL and %CV implying that LB01 and Otto's had more significant efficiencies than the others (Figure 1A and Table 1). Moreover, no detectable tannic acid or phenolic compound effects in the solutions prepared from embryo tissues.

With the oil palm seedlings, the differences were due to different yields, debris, fluorescence intensity, %CV and peak positions observed while analysis of all buffers with one to twelve months-old seedlings. WPB buffer yielded approximately 314 for the first highest FL value and lowest of %CV (8.75%) of one month-old seedlings. The second lowest and highest of %DF and YF were also found with this buffer for 99.74% and 0.27 nuclei s^{-1} mg^{-1}, respectively. Using LB01, the sufficient amount of yields and debris factors were revealed with the first highest and lowest for 0.52 nuclei s^{-1} mg^{-1} and 98.61%, respectively. Using WPB, the peak position of PI-fluorescence intensity from young leaves of one month-old seedling resembled sustainability with those obtained from analysis of embryos by using LB01 (Figure 1A and B). The most suitable buffer for one month-old seedling of oil palm is therefore WPB buffer by means of the highest of FL, lowest of %CV values (Table 1) and maintainable of histogram position.

Figure 1C outlines the PI-fluorescence histograms of three months-old seedlings of oil palm estimated with the five buffers. Only LB01 and WPB buffers affected the sufficient amount of FL and YF and against %CV and DF for this stage of oil palm seedlings. The buffers yielded sufficiently several parameters; the first highest of FL and lowest of %CV were detected in the nuclei solution of WPB (281.17 and 6.45%) whereas %DF and YF were compared to the first lowest (93.41%) and highest (1.81 nuclei s^{-1} mg^{-1}) values in the solution of LB01 (Table 1). In addition the second levels of these parameters were also found correspondingly in those buffers. The chosen buffers for FCM of three month-old seedlings of oil palm are LB01 and WPB buffers. It is interesting to note that peak position of WPB-derived nuclei revealed in the same position compared to the position of one month-old seedlings-derived nuclei isolated by WPB.

For six months-old seedlings, although LB01 produced the first lowest of %DF (96.44%) and the second highest of YF (1.38 nuclei s^{-1} mg^{-1}), the result showed that WPB buffer is the suitable isolation for nuclei extraction by means of the first highest FL (362.28) and lowest %CV (4.47%) values (Figure 1D and Table 1). This result resembled to the analysis of twelve months-old seedlings which showed the same suitable buffer, revealing the first highest FL (285.49) and lowest %CV (6.91%) (Figure 1E and Table 1). Consequently, there are two suitable buffers (WPB and LB01) for analysis of FCM in oil palm by means of the highest of FL and YF and lowest of %CV and DF. However, the position of histogram peaks could be considered significantly because only WPB gave the same position of PI histogram. These seem to be a high efficiency of WPB for maintaining quality of nuclei solution. It is interesting to note that some leaf positions of oil palm seedlings were found to be undetectable tissues for their nuclei isolation when analysis with specific buffer. As above mentioned, the WPB buffer is accepted to estimate 2C-DNA value of oil palm cultivars. Analysis of each buffer on 2C DNA content will be clarified below.

Estimation of nuclear genome size (C-DNA values) of oil palm embryos and seedlings

Table 2 lists the average C-values of 5 growth stages of oil palm determined by using *Glycine* as external reference plant. Using LB01, the 2C DNA contents from oil palm embryos were regularly lessen in width between 3.7-3.8 pg whereas ranging from 2.7-3.0 pg, 4.2-5.2 pg, 2.7-2.8 pg and 1.8-3.1 pg were revealed when using WPB, Otto's, Tris.$MgCl_2$ and Galbraith as nuclear lysis buffers, respectively (data not shown). Although LB01 and Tris.$MgCl_2$ were the most unchangeable buffers for a value of DNA content from FCM, only LB01 is the acceptable nuclear lysis buffer for 2C DNA content value followed by the first lowest value of %CV, the second highest of FL and is resembled with previous reported works (3.7-3.8 pg) (Rival *et al.*, 1997; Srisawat *et al.*, 2005; Madon *et al.*, 2008).

The 2C DNA contents from analysis of one to twelve months-old seedlings of oil palm were significantly different in stages of oil palm seedlings and nuclear lysis buffers, with three positions of their unopened leaves. In one month-old seedlings, with the exception of WPB buffer, the lysis buffers mostly produced the low of 2C DNA content (1.2-2.1 pg). Although leaf-bases of one month-old seedlings did not produce the highest 2C DNA value of oil palm when using WPB buffer, this DNA content closely agreed with our previously reported (3.7 pg) (data not shown). Remarkably, leaf-base of unopened-leaves was found to be most suitable tissue for nuclei isolation. The DNA contents from this tissue were generally higher and more consistent than those obtained from leaf-apices and leaf-middles. Therefore leaf-apices and leaf-middles of oil palm are not suitable tissues for FCM analysis.

For three and twelve months-old seedlings, the results revealed that the average 2C DNA values from analysis with WPB were generally within the range of values obtained in the previous reported for 3.8 pg (Madon *et al.*, 2008). Not only the

WPB buffer found to be the suitable buffer, but also produced the highest values of 2C DNA content. The results of 2C DNA values from three months-old seedlings revealed that using Otto's as nuclear lysis buffer did not extract their nuclei from all leaf positions. In addition both of leaf-apices and leaf-middles were found to be less effective tissues when using Galbraith and Tris.$MgCl_2$ for three and six month-old seedlings of oil palm (Table 2). Since leaf positions used in this work were significant differences of genome size, especially 2C DNA from leaf-bases of six and twelve months-old seedlings higher than those of leaf-apices and leaf-middles, we therefore used leaf-bases of oil palm as the plant material for FCM analysis in cultivars verifying experiment. Moreover, using LB01 (2C DNA content=3.7 pg) and WPB (2C DNA content=3.8 pg) for isolating the nuclei from oil palm embryos and leaves of oil palm seedlings, respectively, resembled those obtained from Rival *et al.* (1997), Srisawat *et al.* (2005) and Madon *et al.* (2008). Therefore, WPB buffer need to be used as the suitable buffer in the next research for cultivars classification in oil palm using young leaves of Dura and Pisifera cultivars as plant materials. In addition, these findings confirmed that DNA content level of oil palm (3.8 pg) could be considered as marker of Tenera for cultivars analysis by using FCM.

Estimation of nuclear genome size (C-DNA values) of Dura and Pisifera cultivars

Young leaves of five parents of Deli Dura and five parents of Pisifera (Calabar, LA ME, DAMI, Nigeria and EKONA) were investigated by using WPB as nuclear lysis buffer. 2C-value of all cultivars revealed differently in terms of DNA content by using Tenera (2C=3.8 pg) as external standard plant. The results showed that DNA contents of Deli Dura (D109, D067, D064, D069 and D068) were ranged from 6.3 - 7.6 pg whereas the DNA content values ranging between 5.3 – 6.1 pg were found in young leaves tissues of Pisifera (Calabar; P109, LA ME; P106, DAMI; P116, Nigeria; P110 and EKONA; P105) (Figure 2 Table 3). Therefore, the numbers of base pairs of one haploid genome of Deli Dura and Pisifera parents were ranged greater than 3000 and 2000 Mbp, respectively.

Discussion

Type of nuclear lysis buffer is known to be a criterion which has been less investigated in restricted reports (Loureiro, *et al.* 2006a, b; Loureiro, *et al.* 2007). Since the investigation of the various FCM nuclear lysis buffers in oil palm has not been described to date, this is therefore the first report on comparison of various lysis buffers with oil palm. It is generally known that there is no individual buffer worked well with all plant species (Loureiro, *et al.* 2006a,b; Loureiro, *et al.* 2007). The effective lysis buffer for FCM of oil palm should be carried out more extensively.

Five buffers used in this study are composed of different types and concentrations of chemical substances. Basically, Tris.$MgCl_2$ is consisted of pH stabilizer (200 mM Tris), preventer of clumping and attachment of debris (0.5% Triton X-100), substance for phenolic compounds bindings (3.0% PVP) and chromatin stabilizer (4 mM $MgCl_2$) whereas another chromatin stabilizer (0.5 mM spermine.4HCl) only found in LB01 buffer. WPB consists differently in some specific substances such as 2 mM Na_2EDTA (chelating agent) and 10 mM sodium metabisulfite (reducing agent which is suppressor of phenolic compound reactions). Polyphenol oxidase inhibitor (catalyse the oxidation of phenolic compounds) is acidulant such as citric acid or sodium citrate found in Otto's and Galbraith buffers, respectively. These acids are used for prevention of phenolic compound reactions. 20 mM MOPS is one type of pH stabilizer that found in Galbraith buffer (Loureiro *et al*, 2007).

Phenolic compounds are plant secondary metabolites, involving in the protection of plants against attacking of pathogens or injuring, and are widely distributed in plant-derived product (Altunkaya and Gokmen, 2009). In this study, physical damage during the chopping process on leaves tissue of oil palm caused an increase of phenolic compound reaction, which often results in color degradation of the tissue or buffer solution from green to brown color. Comparing to chemical compositions on five nuclear lysis buffers, although WPB and Otto's buffers consisted of some reducing agents namely sodium metabisulfite and citric acid, respectively, only Otto's-derived nuclei solution was found to be browning. This implies that sodium metabisulfite in WPB had a positive effect for the prevention of oxidation of phenolic compounds. The effectiveness of this reducing agent could suppress some phenolic compounds releasing when chopping leaf tissues of oil palm in a buffer solution, resulting in consistency of their peak positions, FCM parameters and 2C DNA contents. As described above, the necessary substances in an applied buffer are an effective reducing agent namely sodium metabisulfite (in WPB) resulting in high value of FL, low value of %CV and consistency of PI histogram and an effective chromatin stabilizer namely spermine 4.HCl (in LB01) which resulting in high value of YF and low value of %DF.

After analyses of embryos and one to twelve months-old seedlings of oil palm with the five nuclear lysis buffers, the results confirmed that some phenolic compounds are regularly found in the nuclei extracting solution of seedlings than that of embryos. Because the seedlings gave the higher value of %CV than embryos, it is indicating that "the tannic acid effect" could cause effect on fluorescence histograms of oil palm seedling with higher %DF and higher %CV (Loureiro *et al.*, 2007). Loureiro *et al.* (2006a) analyzed the SS and CV values of *Pisum sativum* nuclei in the solution of Tris.$MgCl_2$ supplemented with various concentrations of tannic acid (TA), a common phenolic compound, at a concentration of 0.75 mL^{-1} to 1.00 mL^{-1} and showed poor reading in peak positions and qualities which might be affected from the role of their phenolic compounds. This reaction is termed that "the tannic effect" by Loureiro *et al.* (2006a).

The buffer given the higher of FL and YF and lower %CV and DF, and gave consistency of PI histograms should be selected for genome size analysis in cultivars verification. After analysis of five nuclear lysis buffers with embryos and seedlings of oil palm, the results revealed significant differences among the buffers and growth stages of oil palm used. Table 2 lists the average C-values of embryos and seedlings of oil palm. Using LB01, 2C DNA content of oil palm embryos was found to be 3.7 pg. Although the flow cytometer, reference plant and type of buffer were used differently, this value resembled those obtained from Rival *et al.* (1997) (FACScan, *Petunia hybrida*, LB01) and Srisawat *et al.* (2005) (FACScalibur, *G. max* cv. Polanka, Tris.$MgCl_2$) for 3.7 pg and mostly closed for 3.8 pg (Madon, *et al.*, 2008; FACScalibur, *G. max* cv. Polanka, LB01). The 2C DNA contents of other buffers have wide ranged from 2.25 - 4.88 pg, resulting in differences type of chemical compositions in each buffer and its concentration. When oil palm tissues were analyzed differently in various growth stages, buffers utilization gave the different reading in peak position among seedlings and its embryos, indicating that some interference occurred between the chopping and staining by phenolic compounds in those seedlings. Using LB01, Tris.$MgCl_2$, Otto's and Galbraith showed less 2C DNA contents of seedlings than that of embryos whereas higher 2C DNA content is only found in WPB which might be affected from the preventer of phenolic compounds reaction (sodium metabisulfite) in WPB solution. The 2C DNA contents of oil palm seedlings ranged differently between 1.95 – 2.76 pg, 3.81 - 4.91 pg, 2.08 – 3.10 pg, 0.75 - 3.46 pg and 0.55 – 1.94 pg when using LB01, WPB, Otto's, Tris.$MgCl_2$ and Galbraith as nuclear lysis buffer compared to that of embryos, respectively. By the way, WPB gave the interesting value of 2C DNA content (3.8 pg) on three and twelve month-old seedlings of oil palm.

For leaf position, 2C DNA contents ranged from 1.47 - 2.90 pg for leaf-apices whereas the DNA contents ranged from 1.09 - 5.48 pg and 0.55 – 4.75 pg when analyzed with leaf-middles and leaf bases, respectively (data not shown). Consequently, there appears to be a close relation between the ability of nuclei isolation and unopened-leaf positions. Moreover the average DNA content of leaf-bases of unopened leaves found to be 3.89 pg (data not shown). Leaf-base is therefore the chosen leaf position for the unopened leaves of oil palm seedlings because this tissue is easier in nuclei isolation and freshness and is suitable for isolating their nuclei in various types of isolation buffers. Furthermore, the interaction among growth stages of seedlings and types of isolation buffers are clarified for the suitable estimation of 2C DNA content analysis of oil palm, indicating the most suitable buffer and tissues for cultivar classification experiment.

For cultivars verification, young leaves of five parents Deli Dura and Pisifera excised from adult tree (unopened leaves) were used as source of nuclei. Although, the DNA contents of Deli Dura (D109, D067, D064, D069 and D068) and Pisifera (Calabar; P109, LA ME; P106, DAMI; P116, Nigeria; P110 and EKONA; P105) cultivars were not significantly differences but it should be distinctly separated into two groups. First is ranged higher than 6.3 pg (Deli Dura) and lower than 6.1 pg (Pisifera) is the second. These data were evaluated and compared to that of their hybrid. It is interesting to note that DNA content of *Tenera* reported here was found to be clearly significantly lower than that of their parents. These resembled the results of using RAPD marker reported by Sathish and Mohankumar (2007) which revealed that Dura showed the highest amounts of bands whereas giving the lowest was found in

Tenera by using an effective marker: P15. The Deli Dura and Pisifera DNA contents analyzed with WPB buffer using Tenera as external standard (3.8 pg) are firstly reported on using progeny DNA value to determined DNA values of their parents. The DNA content of five Deli Dura and five Pisifera parents can be used as a database in any further oil palm breeding programs and may be helpful in verifying cultivars or clone.

In conclusion, we have the possibility to carry out DNA contents of Deli Dura and Calabar, LA ME, DAMI, Nigeria and EKONA Pisifera using DNA content of their hybrid as an external reference plant. The DNA contents of Tenera, Deli Dura and Pisifera are 3.8, 6.3-7.6 and 5.3-6.1 pg, respectively. These DNA contents of oil palm mostly a useful database for routine breeding program and for routine assay of accepted cultivated cultivars. Further application of nuclear lysis buffer for FCM of oil palm should be re-consisted of some effective substances such as sodium metabisulfite and spermine.4HCl for giving the higher of FL and lower of %CV and giving higher of YF and lower of % DF, respectively.

Table 1. Flow cytometric parameters assessed of **oil palm embryos and unopened-leaves of one to twelve month-old seedling** of oil palm in various isolation buffers

Growth stages	**Buffer**	**FCM Parameters (Mean ± SD)**					
		FS (channel unit)	**SS (channel unit)**	**FL (channel unit)**	**CV (%)**	**DF (%)**	**YF (nuclei $s^{-1}mg^{-1}$)**
Embryos	**LB01**	4149.60 ± 2950.15^{a}	862.30 ± 504.36^{a}	**299.73** ± 4.98^{ab}	**4.57** ± 0.38^{a}	99.22 ± 0.15^{a}	0.68 ± 0.36^{a}
	WPB	6723.60 ± 4975.76^{a}	765.77 ± 366.31^{a}	211.36 ± 10.59^{bc}	6.59 ± 2.15^{ab}	99.13 ± 0.59^{a}	**0.99** ± 0.78^{a}
	Otto's	6854.67 ± 5155.52^{a}	2738.87 ± 2078.82^{a}	**385.55** ± 52.42^{a}	**4.93** ± 0.38^{a}	99.08 ± 0.35^{a}	0.78 ± 0.12^{a}
	Tris.$MgCl_2$	6181.40 ± 4571.84^{a}	1582.60 ± 1037.63^{a}	208.91 ± 14.46^{bc}	7.31 ± 0.30^{ab}	**98.93** ± 0.09^{a}	0.96 ± 0.26^{a}
	Galbraith	5860.37 ± 4286.90^{a}	1163.43 ± 585.23^{a}	166.33 ± 60.00^{c}	8.93 ± 2.11^{b}	**98.90** ± 0.33^{a}	**0.96** ± 0.86^{a}
One month-old seedlings	**LB01**	861.93 ± 136.86^{a}	496.63 ± 218.05^{a}	156.30 ± 72.84^{b}	**11.22** ± 3.73^{a}	**98.61** ± 1.09^{a}	**0.52** ± 0.26^{a}
	WPB	919.73 ± 39.66^{a}	593.13 ± 152.19^{a}	**314.65** ± 38.29^{a}	**8.75** ± 3.48^{a}	**99.74** ± 0.17^{a}	**0.27** ± 0.13^{ab}
	Otto's	944.00 ± 113.46^{a}	672.70 ± 434.13^{a}	**159.87** ± 67.45^{ab}	12.87 ± 11.39^{a}	99.89 ± 0.06^{a}	0.10 ± 0.05^{b}
	Tris.$MgCl_2$	*666.00 ± 576.86^{a}	*457.50 ± 446.86^{a}	*72.74 ± 64.19^{b}	*8.36 ± 7.26^{a}	*64.87 ± 56.25^{a}	*0.03 ± 0.03^{b}
	Galbraith	1019.40 ± 1.25^{a}	1009.533 ± 7.98^{a}	94.16 ± 37.32^{b}	18.86 ± 5.77^{a}	99.94 ± 0.01^{a}	0.07 ± 0.03^{b}

	LB01	707.70 ± 250.07^{a}	273.27 ± 220.98^{a}	**203.72** ± 85.90^{a}	**6.52** ± 3.89^{bc}	**93.41** ± 10.08^{b}	**1.81** ± 2.46^{a}
	WPB	881.87 ± 49.47^{a}	263.80 ± 143.24^{a}	**281.17** ± 21.57^{a}	**6.45** ± 1.56^{bc}	**99.80** ± 0.06^{b}	**0.21** ±0.05^{a}
Three month-old seedlings	Otto's	-	-	-	-	-	-
	Tris.$MgCl_2$	911.03 ± 56.91^{a}	233.60 ± 137.67^{a}	59.12 ± 6.17^{b}	12.86 ± 1.72^{c}	99.82 ± 0.02^{b}	0.15 ± 0.03^{a}
	Galbraith	*272.83 ± 472.56^{ab}	*141.57 ± 245.20^{a}	*14.18 ± 24.55^{b}	*1.63 ± 2.82^{ab}	*32.65 ± 56.55^{ab}	*0.41 ± 0.71^{a}
	LB01	658.13 ± 249.40^{ab}	326.37 ± 295.42^{a}	223.24 ± 24.34^{ab}	**6.38** ± 1.01^{ab}	**96.44** ± 2.17^{a}	**1.38** ± 0.66^{ab}
	WPB	922.20 ± 24.53^{a}	365.93 ± 227.66^{a}	**362.78** ± 44.11^{a}	**4.47** ± 1.65^{ab}	99.59 ± 0.26^{a}	0.50 ± 0.30^{ab}
Six month-old seedlings	Otto's	863.10 ± 49.96^{a}	306.53 ± 196.14^{a}	**228.19** ± 106.14^{ab}	7.18 ± 1.30^{ab}	99.72 ± 0.08^{a}	0.23 ± 0.10^{b}
	Tris.$MgCl_2$	*179.10 ± 310.21^{b}	*18.37 ± 31.81^{a}	*94.15 ± 163.07^{b}	*2.47 ± 4.28^{a}	*33.28 ± 57.65^{a}	*0.05 ± 0.09^{b}
	Galbraith	853.40 ± 179.79^{a}	538.93 ± 319.73^{a}	161.35 ± 12.76^{ab}	10.11 ± 1.22^{b}	**97.03** ± 1.96^{a}	**1.74** ± 0.87^{a}
	LB01	466.70 ± 103.06^{a}	147.93 ± 116.39^{a}	164.34 ± 41.55^{bc}	10.28 ± 5.14^{a}	**97.57** ± 0.12^{a}	**0.83** ± 0.23^{a}
	WPB	909.67 ± 34.12^{a}	224.33 ± 57.91^{a}	**285.49** ± 28.90^{a}	**6.91** ± 2.65^{a}	99.71 ± 0.17^{a}	**0.34** ± 0.14^{a}
Twelve month-old seedlings	**Otto's**	720.23 ± 63.31^{a}	142.43 ± 55.40^{a}	**238.15** ± 25.23^{ab}	**8.41** ± 3.06^{a}	**99.59** ± 0.33^{a}	0.27 ± 0.23^{a}
	Tris.$MgCl_2$	470.17 ± 98.76^{a}	141.43 ± 48.14^{a}	201.03 ± 24.71^{ab}	7.33 ± 0.55^{a}	99.70 ± 0.09^{a}	0.21 ± 0.06^{a}
	Galbraith	*425.50 ± 378.34^{a}	*395.30 ± 426.18^{a}	*83.11 ± 72.02^{c}	*8.63 ± 7.60^{a}	*64.73 ± 56.12^{a}	*1.11 ± 0.98^{a}

Values are given as mean and standard deviation of the mean (SD) of forward scatter (FS, channel units), side scatter (SS, channel units), fluorescence (FL, channel units), coefficient of variation of G0/G1 DNA peak (CV, %), debris background factor (DF, %) and yield factor (YF, %).

Means for the same growth stages followed by the same letter (a, b or c) are not statistically different according to the multiple comparison Tukey test at $P \leq 0.05$.

The parameter's first and second highest (FL and YF) and lowest (CV and DF) values of the estimation in each essential parameter are shown in bold type.

The nuclear lysis buffer chosen for the FCM of oil palm embryos and one to twelve month-old seedlings of oil palm are shown in bold type.

* Buffer did not produce their nuclei from some leaf positions.

- No oil palm nuclei peaks were distinguishable.

Table 2. Estimation of genome size in oil palm embryos and one to twelve month-old seedlings of oil palm using *Glycine max* cv. Polanka (2C=2.5 pg) as external reference plant

Growth stages	Buffers	C-DNA values (Mean ± SD)		
		2C (pg)	**1C (Mbp)**	**Peak CV(%)**
Embryos	**LB01**	**3.75 ± 0.06^{b}**	1834	4.57 ± 0.38^{a}
	WPB	2.91 ± 0.15^{bc}	1422	6.59 ± 2.15^{ab}
	Otto's	4.88 ± 0.57^{a}	2389	4.93 ± 0.38^{a}
	Tris.$MgCl_2$	2.80 ± 0.04^{bc}	1370	7.31 ± 0.30^{ab}
	Galbraith	2.25 ± 0.71^{c}	1100	8.93 ± 2.11^{cb}
One month-old seedlings	LB01	1.95 ± 0.91^{b}	954	11.22 ± 3.73^{a}
	WPB	4.30 ± 0.54^{a}	2103	8.75 ± 3.48^{a}
	Otto's	2.08 ± 0.88^{b}	1017	12.87 ± 11.39^{a}
	Tris.$MgCl_2$	*1.38 ± 0.22^{b}	675	8.36 ± 7.26^{a}
	Galbraith	1.22 ± 0.48^{b}	597	18.86 ± 5.77^{a}

Three month-old seedlings	LB01	2.44 ± 1.09^{a}	1193	6.52 ± 3.90^{ab}
	WPB	**3.81 ± 0.30^{a}**	1863	6.45 ± 1.56^{ab}
	Otto's	-	-	-
	Tris.$MgCl_2$	0.75 ± 0.43^{b}	367	12.86 ± 1.72^{a}
	Galbraith	*0.55	269	1.63 ± 2.82^{ab}
Six month-old seedlings	LB01	2.76 ± 0.31^{b}	1350	6.38 ± 1.01^{b}
	WPB	4.91 ± 0.56^{a}	2401	4.47 ± 1.65^{b}
	Otto's	3.10 ± 1.44^{ab}	1516	7.18 ± 1.30^{ab}
	Tris.$MgCl_2$	*3.46	1692	2.47 ± 4.28^{b}
	Galbraith	1.94 ± 0.16^{b}	949	10.11 ± 1.22^{a}
Twelve month-old seedlings	LB01	2.02 ± 0.51^{bc}	988	10.28 ± 5.14^{a}
	WPB	**3.82 ± 0.39^{a}**	1868	6.91 ± 2.65^{a}
	Otto's	2.99 ± 0.31^{ab}	1462	8.41 ± 3.06^{a}
	Tris.$MgCl_2$	2.71 ± 0.33^{b}	1325	7.33 ± 0.55^{a}
	Galbraith	*1.57 ± 0.04^{c}	768	8.63 ± 7.60^{a}

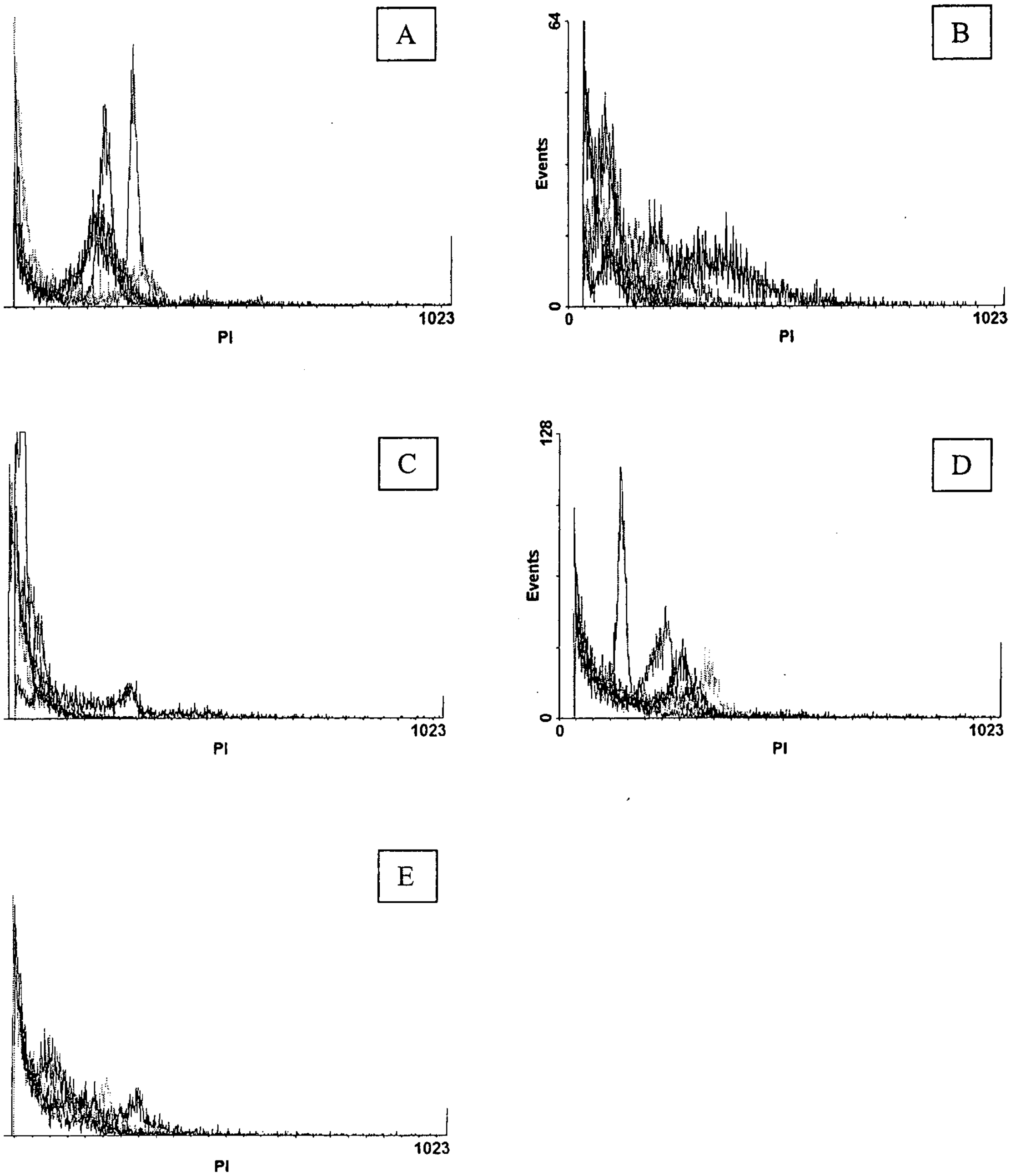
Values are given as mean and standard deviation of the mean genome in mass values (2C, pg) and base pair (1C, Mbp), 1 pg = 978 Mbp (Dolezel *et al.*, 2003).

Means for the same growth stages followed by the same letter (a b or c) are not statistically different according to the multiple comparison Tukey test at $P \leq 0.05$.

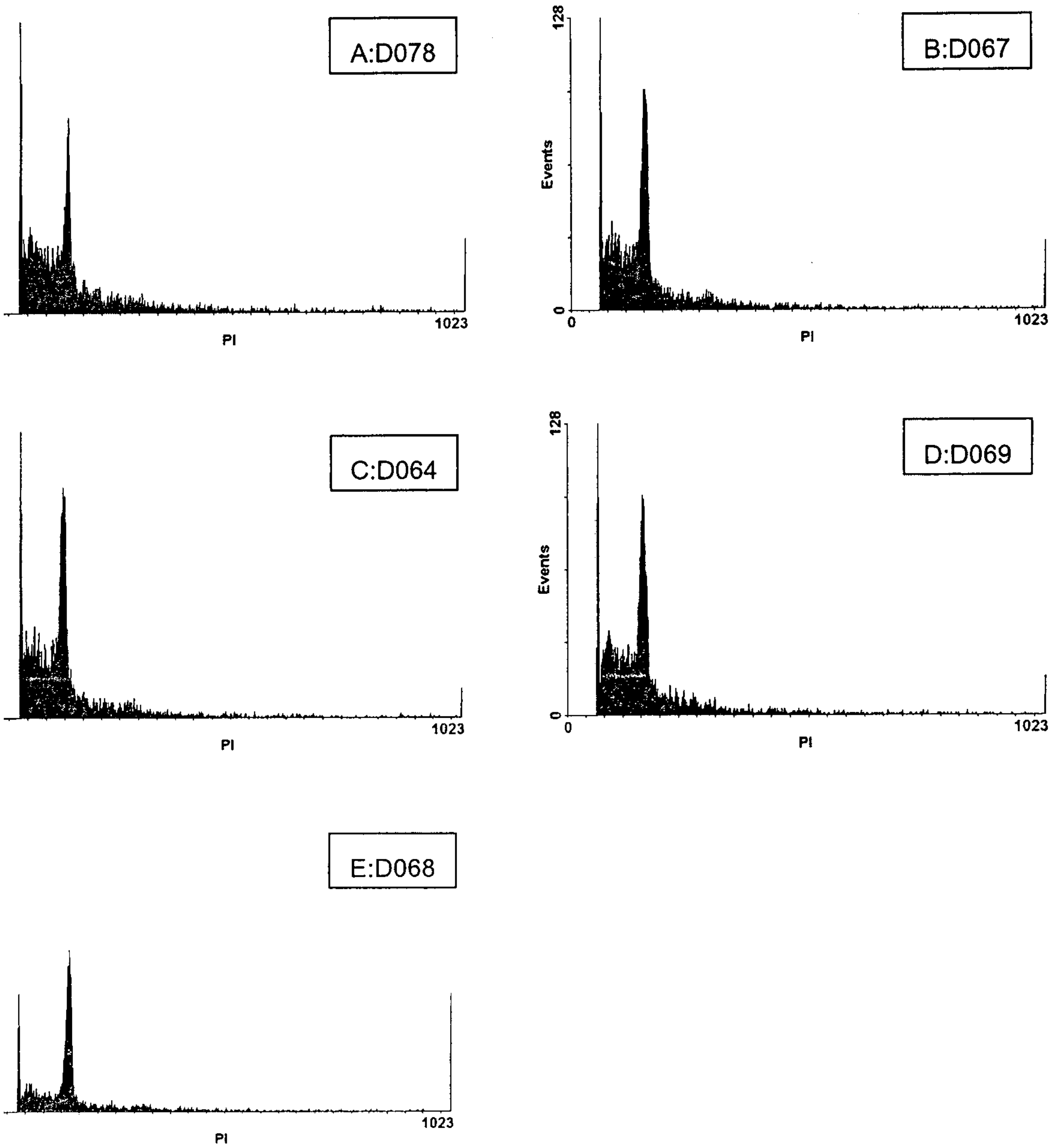
The nuclear lysis buffer chosen for the FCM of oil palm embryos and three and twelve month-old seedlings of oil palm are shown in bold type.

* Buffer did not produce their nuclei from some leaf positions.

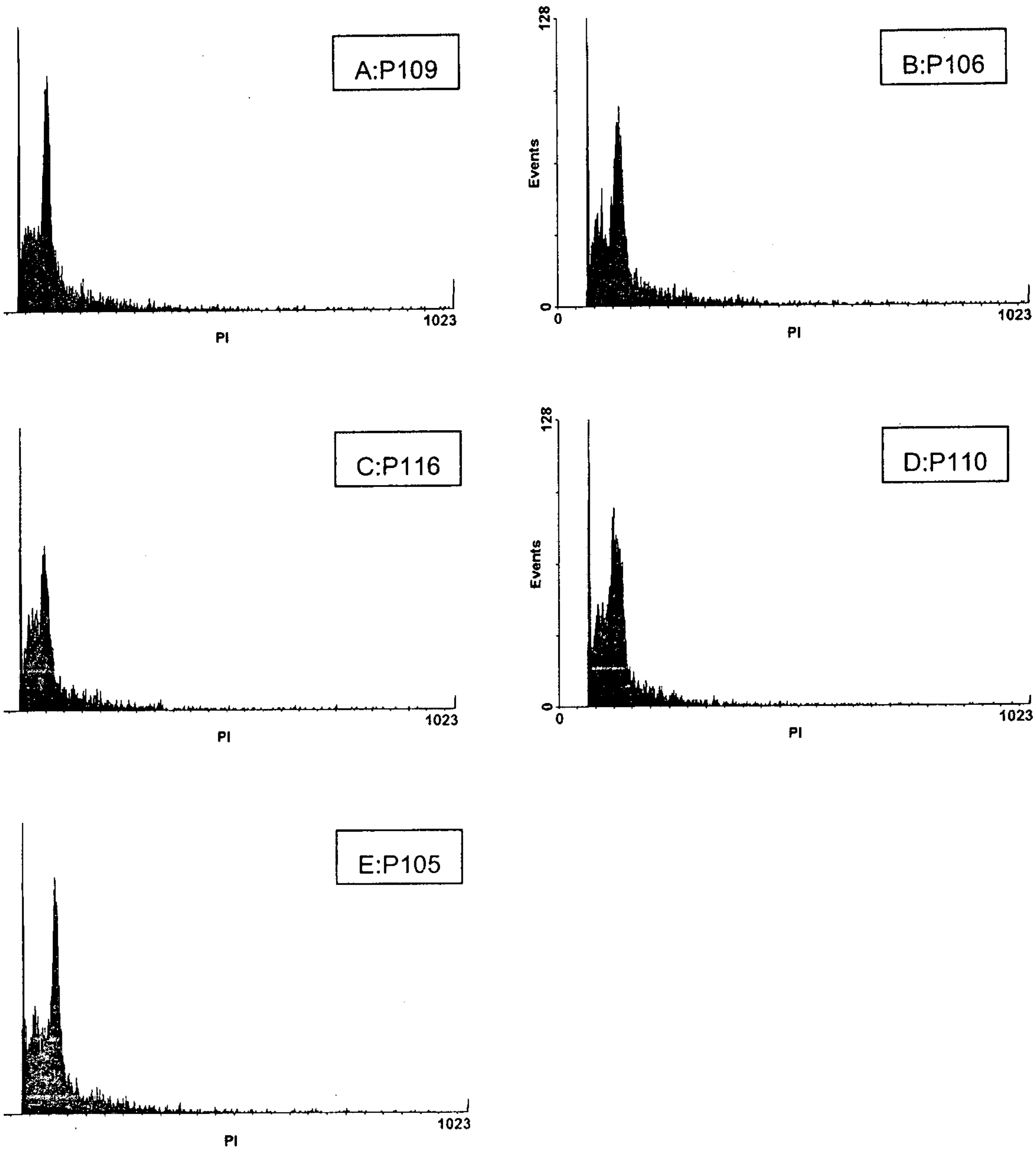
- No oil palm nuclei peaks were distinguishable.



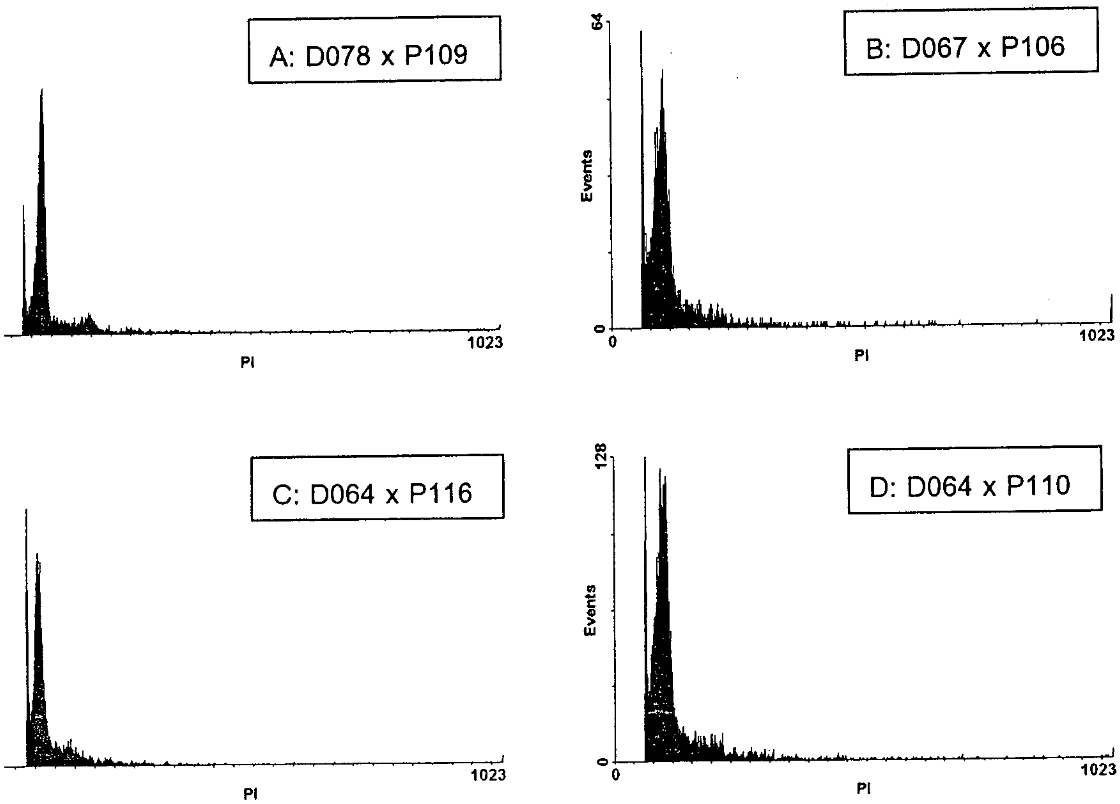
. Histogram of relative fluorescence intensities (PI fluorescence) of oil palm embryo nuclei
ne month-old seedling (B), three months-old seedling (C), six months-old seedling (D) and
e months-old seedling (E) isolated with five nuclear lysis buffers. (LB01 buffer: Red, WPB
r: Black, Tris.MgCl2 buffer: Blue, Otto's buffer: Green and Galbraith buffer: Purple)



. A-E Histogram of relative fluorescence intensities (PI fluorescence) of five parents of Deli , nuclei of young leaves isolated with WPB buffer



'. A-E Histogram of relative fluorescence intensities (PI fluorescence) of five parents of
ɜra; Calabar: P109 (A), LA ME: P106 (B), DAMI: P116 (C), Nigeria: P110 (D) and
NA: P105 (E), nuclei of young leaves isolated with WPB buffer .



ι. A-D Histogram of relative fluorescence intensities (PI fluorescence) of four hybrid ·ra; Suratthani 1 (A), Suratthani 2 (B), Suratthani 3 (C) and Suratthani 5, nuclei of young ;s isolated with WPB buffer

Table 3. Estimation of genome size (C-DNA values) in five Deli Dura and five Pisifera parents

Hybrid Tenera	Cultivars (code)	C-DNA values (Mean ± SD)		
		2C (pg)	1C (Mbp)	Peak CV (%)
Suratthani 1	Deli Dura (D078)	6.88±0.67^{ab}	3362	3.84±0.49^{ab}
	Calabar Pisifera (P109)	6.14±0.74^{ab}	3005	4.58±0.49^{ab}
Suratthani 2	Deli Dura (D067)	7.13±0.27^{ab}	3484	4.17±0.45^{ab}
	LA ME Pisifera (P106)	5.96±0.66^{ab}	2915	6.13±1.63^{a}
Suratthani 3	Deli Dura (D064)*	6.70±0.23^{ab}	3278	4.52±0.62^{ab}
	DAMI Pisifera (P116)**	5.37±0.72^{b}	2628	6.08±1.75^{ab}
Suratthani 4***	Deli Dura (D069)	6.35±0.07^{ab}	3103	3.80±0.34^{ab}
	EKONA Pisifera (P105)	5.44±0.62^{b}	2659	5.08±1.35^{ab}
Suratthani 5	Deli Dura (D064)*	6.70±0.23^{ab}	3278	4.52±0.62^{ab}
	Nigeria Pisifera (P110)	5.90±1.09^{ab}	2884	4.81±1.58^{ab}
Suratthani 6***	Deli Dura (D068)	7.62±0.14^{a}	3725	3.30±0.14^{b}
	DAMI Pisifera (P116)**	5.37±0.72^{b}	2628	6.08±1.75^{ab}

Values are given as mean and standard deviation of the mean genome in mass values (2C, pg) and base pair (1C, Mbp), 1 pg = 978 Mbp (Dolezel *et al.*, 2003) using *Elaeis* cv. Tenera (2C=3.8 pg) as external reference plant

Means for the same growth stages followed by the same letter (a b or c) are not statistically different according to the multiple comparison Tukey test at $P \leq 0.05$.

* Tenera Suratthani 3 used the same Deli Dura with Hybrid Tenera Suratthani 5

**Tenera Suratthani 3 used the same DAMI Pisifera with Hybrid Tenera Suratthani 6

***No longer available of seed production.

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