ANALYSIS OF OIL PALM CALLI AND REGENERANTS USING FLOW AND IMAGE CYTOMETRY AND 18S-25S RIBOSOMAL DNA FLUORESCENCE *in situ* HYBRIDISATION (FISH)

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ABSTRACT

Flow (FCM) and image cytometry (ICM) and 18S-25S ribosomal DNA (rDNA) fluorescence in situ *hybridisation (FISH) were used to analyse the genetic stability or variation in oil palm* in vitro *plantlets, various forms of calli, and slow- and fast-growing liquid suspension cultures. Both FCM and ICM results show a similar correlation pattern where samples with lower DNA content had lower integrative optical density (IOD) peaks. The 18S-25S rDNA FISH showed two large signals in the interphase cells of calli analysed, indicating a diploid ploidy level. Significant differences were observed in the DNA content and pattern of nuclei activity of the slow- and fast-growing liquid suspension cultures using FCM and ICM analysis, demonstrating the usefulness of the above-mentioned tools developed to analyse materials prior to micropropagation; hence, the oil palm's clonal fidelity is ensured, and the efficiency and robustness of liquid culture are improved by selecting callus materials which have similar DNA content and IOD range and peak as the fast-growing friable suspension and friable calli used in this study.*

INTRODUCTION

Oil palm (*Elaeis guineensis*) is a major crop widely grown across the tropics, and is planted on more than 4.7 million hectares of land in Malaysia. Commercially used planting materials are F_1 hybrids of *dura* (having large fruits with thick shells, and a small proportion of oil-bearing mesocarp) and *pisifera* (with shell-less fruits and are essentially female-sterile) called *tenera* (bearing smaller fruits with a thinner shell, smaller kernels and a larger proportion of the oil-bearing mesocarp compared to *dura*) (Moretzsohn *et al.*, 2000). The switch to planting *tenera* instead of *dura* has resulted in yield improvements of up to 30%. Oil palm is heterogeneous so clonal propagation of elite palms with good traits such as high yield of fresh fruit bunches (FFB), high monounsaturated oil, short trunks or bigger fruit types is desirable (Hardon *et al.*, 1987). High-yielding genetic materials have been reported where the best experimental plot produced some 8.6 t FFB ha^{-1} yr^{-1} (Corley *et al.*, 1976), selected progeny produced 12.2 t ha^{-1} yr^{-1} (Rajanaidu *et al.*, 1990), individual palms yielded 13.6 t ha^{-1} yr^{-1} (Sharma and Tan, 1999) and the maximum theoretical yield of 18.2 t ha^{-1} yr^{-1} has been estimated (Corley, 1998). These outstanding palms can be cloned to provide high-yielding planting materials for the industry.

Oil palm cloning protocols were established in the 1970s (Jones, 1974; Rabéchault and Martin, 1976; Paranjothy and Othman, 1982). Explants used in the early years were zygotic embryos (Jones, 1974); later, leaf tissues from 'cabbages' (very young leaves taken from above the apical meristem) of mature elite palms (the ortets) were utilised, these being tissues that are abundant and microbiologically sterile (Rohani *et al.*, 2003). Oil palm clonal abnormality

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was first reported in the mid-1980s (Corley *et al.,* 1986), causing major havoc among oil palm tissue culturists. Therefore, a better understanding of the nature and effects of tissue culture procedures, including understanding genomics and molecular biology, is needed as it underpins biotechnology applications in the crop. Clearly, the events are not a simple 'dedifferentiation' but a complex series of many parts of normal plant development that lead to the differentiation pathways in tissue culture.

It has been reported that the performance of oil palm cultures is genotype-dependent and that the duration for establishment varies from clone to clone (Tarmizi, 2002). Furthermore, depending on the tissue culture processes, media and clones, various types of calli can be produced. Despite the existence of a global collection of oil palm germplasm which has high genetic and allelic diversity [for example, the African germplasm collected by MPOB and analysed via isoenzyme profiling (Hayati *et al.*, 2004)], the commercial plantations have a very narrow genetic base with a severely limited number of *pisifera* male parents.

Information on genome size variation of calli and regenerants has been reported by Jones *et al*. (1982) and Rival *et al*. (1997). These researchers used micro-densitometry and flow cytometry, respectively, to estimate the DNA content of various calli and regenerants. Our study was undertaken to analyse the various types of calli and regenerants originating from different clones via flow and image cytometry and 18s-25s rDNA fluorescence *in situ* hybridisation (FISH). Flow cytometry was used to estimate the genome size of the different calli and leaves from clonal plantlets. This technique enables the measurements to be made on thousands of nuclei. To complement this, image cytometry with Feulgen fluorescence was used to analyse individual nuclei, and integrative optical density (IOD) measurements were done on at least one hundred nuclei whenever possible. It is felt that

the two technologies complement each other where the thousands of nuclei that pass through flow cytometry can be visualised cytologically and examined extensively by image cytometry. FISH of 18s-25s rDNA was used mainly to ascertain the ploidy level where the number of rDNA signals in the unexpressed mRNA state of interphase nuclei indicates the ploidy state regardless of genome size.

MATERIALS AND METHODS

Plant Materials

Oil palm calli were initiated from immature leaves of various ortets from the MPOB collections [clone E63 (ortet 0.189/2844), clone E73 (ortet 0.189/2677) and clone E85 (ortet 0.150/2677)] cultured on solid MS (Murashige and Skoog, 1962) medium with 1 mg litre^{-1} nicotinic acid, 0.1 g litre^{-1} myoinositol, 0.1 g litre^{-1} L-glutamine, 3% sucrose and supplemented with 186 mg litre^{-1} NAA + 2500 mg litre^{-1} activated charcoal (AC) (Rohani *et al.*, 2003). For establishment of liquid cultures, the selected calli were transferred to liquid MS media supplemented with 1 mg litre^{-1} 2,4-D and 0.1 mg litre^{-1} NAA without AC. Other components remained the same as in the solid culture medium (Tarmizi, 2002). The leaf and liquid cultures (100 rpm on an orbital shaker) were incubated in darkness while rooted plantlets were maintained in 12-hr light photoperiod. Each shelf was illuminated by two 1.2 m long fluorescent lamps (36-Watt). Room temperature for both the lit and dark rooms was maintained at 28°C $\pm$ 2°C. For flow and image cytometry analysis, young leaves and root tips of soyabean (*Glycine max* cv 'Polanka') were used as standards (2C=2.5 pg; seeds were kindly provided by J Dolezel, Oloumec, Czech Republic) while the oil palm samples used are listed in *Table 1*.

TABLE 1. SAMPLES USED FOR FLOW AND IMAGE CYTOMETRY AND FLUORESCENCE *in situ* HYBRIDISATION (FISH) OF RIBOSOMAL DNA (rDNA)

Flow cytometry	Image cytometry	FISH of 18S.25S rDNA
Tenera clone E63 first leaf	*Tenera* clone E63 root tips	-
Tenera clone E85 first leaf	*Tenera* clone E85 root tips	-
Friable suspension calli E73, slow-growing (FSC E73-SG)	FSC E73-SG	-
Friable suspension calli E63, fast-growing (FSC E63-FG)	FSC E63-FG	FSC E63-FG and cells from supernatant fraction
Friable suspension calli E85, fast-growing (FSC E85-FG)	FSC E85-FG	-
Rooty suspension calli (RSC) E63	RSC E63	-
Friable calli (FC)	FC	FC
Nodular calli (NC)	NC	NC
Rooty calli (RC)	RC	-

Flow Cytometry Analysis

Nuclei were extracted by placing approximately 50 mg sliced fresh leaf tissues or calli into a 50 µm micro tissue homogeniser (Medicon, Becton Dickinson) containing 1 ml LBO1 lysis buffer, made up of 15 mM Tris, 2 mM Na$_2$EDTA, 80 mM KCl, 20 mM NaCl, 0.5 mM spermine, 15 mM mercaptoethanol, 0.1% Triton X-100, pH 7.5 with 50 µg propidium iodide and 50 µg RNaseA (Dolezel *et al.*, 1989;). The samples were blended for 3 to 5 min; suspended nuclei were removed using a 1-ml syringe and filtered through a 50-µm filcon (Becton Dickinson) into a 10-ml tube. The filtrates were analysed using a flow cytometer (Becton Coulter, at Leicester Royal Infirmary Hospital, United Kingdom), equipped with an argon ion laser (15 mW) at 488 nm. Fluorescent intensity histograms were collected over 1024 channels, and for each sample 10 000 events were captured. Three replicates were carried out for each sample and standard. *Table 2* shows the values of fluorescence intensities associated with the different histogram peaks observed. The flow distribution data were analysed using the software WIN MDI 2.8 (by UCLA).

Image Cytometry

Root tips of soyabean (standard) and the various types of calli were fixed in 4% formaldehyde while the root tips of oil palm clonal plantlets were fixed in alcohol:acetic acid (3:1) and stored at 4°C until needed. The image cytometry procedure was modified from the method of Greilhuber and Temsch (2001). First, two sets of samples and standard were rinsed several times in distilled water. The materials were then acid-hydrolysed in 5N HCl for 90 and 120 min at 20°C to observe any differences in acid hydrolysis time. Materials were then rinsed in ice-cold distilled water for a maximum of 10 min (5 × 2 min), and immersed in Schiff's reagent at room temperature (for 1.5 hr) or at 4°C (for 12-15 hr or overnight). Next, they were rinsed in sulphide water for 3 × 5 min, followed another rinsing for 3 × 10 min. After a short rinse in distilled water, samples and parallel standards were squashed with 45% acetic acid within 3 to 5 min. Slides were frozen to remove the cover-slips, and dehydrated in an ethanol series of 50%, 70%, 90% and finally 96% for 10 min each. The slides then were mounted with antifade media (Citifluor) and observed using epifluorescent microscopy.

Image Analysis

The images of the cells observed under Feulgen fluorescence (red) were captured using a CCDOPS camera and the black and white images were saved in the TIFF format. All images were captured under the same illumination, exposure and magnification conditions. IOD values of the nuclei were measured by using the Scion Image-Release Beta 4.02 (NIH) software by multiplying the area and mean density values for each nucleus. The histogram charts of IOD associated with the percentage nuclei frequency were constructed using Microsoft Excel to observe the pattern of nuclei activity.

Fluorescence *in situ* Hybridisation of 18S-25S rDNA Probe

The samples used for the FISH experiments were nodular calli, the friable and supernatant fraction of the friable suspension calli. Samples were digested overnight with an enzyme mixture of 2% cellulase and 20% pectinase in an enzyme buffer at 37°C. The suspension was filtered to remove the debris, and centrifuged at 800 g to pellet the protoplast. This was followed by 2 × 5 min wash in the enzyme buffer, and the pellets were finally resuspended in 100 µl 60% acetic acid. For each slide, 10 µl of suspension were dropped onto a pre-cleaned slide, covered with a cover-slip and firm thumb pressure was applied. The slides were then frozen on dry ice, and with the cover-slips flipped off, followed by serial ethanol dehydration, air-drying and storage at -20°C.

For the FISH experiments, the slides were re-fixed and cleaned in alcohol:acetic-acid (3:1) for 10 min, washed twice in 96% ethanol for 10 min each time, and air dried. 200 µl of RNaseA (100 µg ml^{-1}) were added to the area of the sample on each slide, covered with a large plastic cover-slip and incubated for 1 hr at 37°C in a humid chamber. The cover-slips were then removed and the slides were washed in 2xSSC twice for 5 min, followed by incubation in 10 mM HCl for 5 min. Upon completion, the slides were tipped slightly to remove the fluid, 200 µl of pepsin (10 µg ml^{-1}) were added. Each slide was covered with a plastic cover-slip and incubated for 10-15 min at 37°C in the humid chamber. The slides were then rinsed twice for 5 min in 2xSSC upon completion, placed in 4% paraformaldehyde fixative for 10 min in a fume cupboard and again followed by 2xSSC washes for 5 min. Finally, the slides were dehydrated in an ethanol series (70%, 90% and 96% ethanol, 2 min each) and air-dried.

The hybridisation mixture consisting of 50% formamide, 2x SSC, 10% dextran sulphate, 1 µg salmon sperm DNA, 0.15% SDS, 25-200 ng of labelled probe and 0.25 mM EDTA were prepared for each slide in a total volume of 40 µl. The mixture was denatured at 70°C for 10 min followed by cooling on ice for 5 min, placed onto the preparation and covered with a small plastic cover-slip cut from an autoclavable plastic bag. The slides were then denatured on a flatbed thermal cycler for 10 min at

80°C, followed by incubation overnight at 37°C. The following morning, the slides were washed first in 2xSSC at 42°C-45°C to float off the cover-slips, and washed again with fresh 2xSSC at 42°C for 2 min, in 0.1xSSC at 42°C for 2 and 10 min, and in 2xSSC for 5 min, and finally cooled to room temperature. They were then incubated in a detection buffer (4xSSC, 0.2% Tween 20) for 5 min before 200 µl of 5% BSA in the detection buffer were added, a large plastic cover-slip applied and incubated at 37°C for 30 min. A detection solution was prepared by adding 6 µl of Alexa 594-biotin to 450 µl of 5% BSA block. After incubation, the cover-slips were lifted off and the slides tipped to remove excess fluid. The 50 µl of the detection reagent were added onto the slide, a small plastic cover-slip applied, and again slides were incubated for 1 hr at 37°C in the humid chamber. Next, the slides were washed in the detection buffer 2×5 min and 1×10 min at 42°C, followed by staining with 100 µl of DAPI (4 µg ml^{-1}) per slide for 10 min while avoiding bright light. The slides were then rinsed in the detection buffer before being mounted with antifade (Citifluor), and excess medium was removed by squeezing the slides between several sheets of filter paper. The preparations were analysed with an Axioplan2 imaging epifluorescence microscope, and the images were captured using a ProgResC12 camera and saved in the TIFF format. The images were overlaid using Adobe Photoshop 6.0 by which image brightness, contrast and enhancement only were optimised.

RESULTS AND DISCUSSION

Types of Calli Produced

Micropropagation of oil palm yielded different types of calli, and they were classified as friable (yellowish in colour, *Figure 1a*), nodular (off-white in colour, *Figure 1b*) and rooty (off-white nodular calli with rooty structures and whitish root tips, *Figure 1c*). The friable embryogenic calli were used

as inocula, as an alternative to cultures on solid medium (Tarmizi, 2002), thus, producing friable suspension calli (FSC). Such cultures have potential for automation and scaling-up of propagule production and for improving growth and culture uniformity (de Touchet *et al.*, 1991; Teixera *et al.*, 1995; Wong *et al.*, 1999). For clones, E63 and E85, FSC grew at a much faster rate, hence the regenerants were established earlier *in vitro* compared to the slower growing E73.

Flow Cytometry (FCM) Analysis

FCM analysis was carried out on the *in vitro* plantlets, calli and suspension calli (*Table 1*). The DNA content of clonal plantlet E63 was 2C = 3.29 ± 0.13 pg and of E85 was 2C = 4.04 ± 0.13 pg. These values were significantly different (P<0.05) despite both being fast-growing calli. No comparison was available with E73 due to the slow establishment of its regenerants. Next, DNA estimation and ANOVA were performed on FSC E63-FG (fast-growing) and E73-SG (slow-growing); the size of FSC was important to obtain clear and debris-free histogram peaks. During this analysis, the size of FSC E63-FG and E73-SG was around 3 mm while FSC E85-FG was about 1 mm, giving no clear peak. The estimated DNA content obtained for FSC E63-FG was 2C = 4.63 ± 0.02 pg and for FSC E73-SG was 2C = 5.08 ± 0.12 pg, the values being significantly different (p<0.05). Apparently, the slow-growing calli E73 had a significantly higher DNA content than the fast-growing E63. For RSC E63-FG, the DNA content was 2C = 4.96 ± 0.04, which was significantly higher than the DNA content of FSC E63-FG but lower than that of FSC E73-SG. The increase in DNA in FSC E63-FG may be due to the rooty structures that were attached to the nodular calli. FCM analysis and ANOVA were performed to compare the DNA contents of FC, NC and RC, and the values obtained were 2C = 5.07 ± 0.08, 5.71 ± 0.01 and 5.28 ± 0.21 pg, respectively, while ANOVA indicated significant differences in the DNA content of the three calli types.
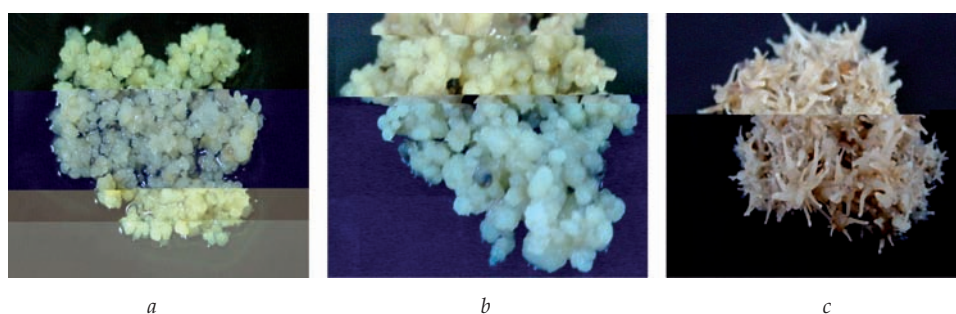

a   *b*   *c*

Figure 1. Different types of calli: (a) friable – yellowish calli, (b) nodular – off-white calli and (c) rooty – nodular calli with root structures complete with whitish root tips.

TABLE 2. ESTIMATED DNA CONTENT, ANALYSIS OF VARIANCE (ANOVA), RANGE OF INTEGRATIVE OPTICAL DENSITY (IOD) PEAK AND PEAK OCCURRENCES FOR THE VARIOUS SAMPLES ANALYSED

Sample	Flow cytometry		Image cytometry	
	2C DNA content (pg)	ANOVA	IOD range	IOD peak
FSC E63-FG	4.63 ± 0.02	Significantly different from one another	40 000-180 000	120 000
FSC E73-SG	5.08 ± 0.12		140 000-500 000	280 000
FSC E85-FG	No peak		40 000-140 000	80 000
RSC E63	4.96 ± 0.04	Significantly different from FSC E63, FSC E73 and rooty calli (RC)	20 000-60 000, 140 000-380 000	
FC	5.07 ± 0.08	Significantly different from one another	140 000-340 000	220 000
NC	5.71 ± 0.01		20 000-80 000, 160 000-400 000, many nuclei with IOD higher than 500 000	220 000, 260 000
RC	5.28 ± 0.21		200 000-300 000, 300 000-500 000, many nuclei with IOD higher than 500 000	220 000, 340 000
Leaf*/root tips of clone E63	*3.29 ± 0.13	Significantly different from one another	40 000-260 000	120 000
Leaf*/root tips of clone E85	*4.04 ± 0.13		100 000-260 000, 260 000-360 000	140 000
Leaf* of *Glycine max* cv. Polanka (standard)	*2C=2.5pg		100 000-380 000, 300 000-400 000, 400 000-500 000, many nuclei with IOD higher than 500 000	220 000, 440 000

Table 2 lists the estimated DNA contents and ANOVA results while *Figure 2* shows some of the samples' histogram peaks.

Rival *et al*. (1997) reported that the relative fluorescence of nuclei was found to be significantly higher in leaves than in calli; however, in our work, the opposite and almost opposite were observed for the FCM and ICM analyses, respectively (*Table 2*). This may be due to the different source of samples, standard and conditions of the lysis buffer used. While we also used LBO1 buffer, Rival *et al*. (1997) used 0.5% Triton X-100 at pH 9.2 whereas we used unmodified LBO1 buffer, pH 7.5 with 50 µg PI and 50 µg RNaseA per 1 ml buffer. The results may also have been due to the differences in chromatin condensation, which is sensitive to even small fluctuations of the nuclear environment (Darzynkiewitz *et al*., 1975; Johnston *et al*., 1996), and to the variations between tissues (Dolezel *et al*., 1989; Vago, 1993; Rival *et al*., 1997). Nuti Ronchi *et al*. (1973) reported the occurrence of extra synthesis of DNA which precedes cell proliferation during the dedifferentiation process of *Nicotiana glauca* pith tissue cultured *in vitro*, while selective loss and regain of chromatin in response to environmental conditions have been described during cell culture and plant regeneration of *Scilla siberica* (Deumling and Clermont, 1989) and *Pisum sativum* (Cecchini *et al*., 1992; Geri *et al*., 1999).

Image Cytometry (ICM) Analysis

Samples that had been acid-hydrolysed for 90 min gave a good fluorescent intensity compared to the too bright fluorescence of samples acid-hydrolysed for 120 min. IOD values of these nuclei were measured (samples in *Table 1*) and histogram charts of IOD associated with the percentage nuclei frequency were constructed to observe the patterns of nuclei activity. *Figure 3* shows examples of histogram charts of IOD bins associated with the nuclei frequency of the root tips of the *tenera* clone E63, E63 fast-growing friable suspension calli (FSC E63-FG) and the supernatant fraction of FSC E63-FG nuclei. *Table 2* lists the DNA content values, ANOVA results, IOD range and peaks obtained from the ICM studies. It was observed that ICM showed a similar pattern to the FCM measurements where a lower IOD peak showed a lower 2C value. For example, FSC E63-FG exhibited 2C IOD peak at 120 000 units with DNA content 2C = 4.63 ± 0.02 pg while FSC E73-SG exhibited 2C IOD peak at 280 000 units with DNA content 2C = 5.08 ± 0.12 pg. Using ICM analysis, a pattern could be observed for FSC E85-FG where IOD ranged from 40 000-140 000 units with the 2C peak occurring at 80 000 units, notwithstanding the small 1 mm calli which restricted analysis via FCM.

a. First leaf of clone E63.

b. Friable suspension calli of E63.

c. Friable calli
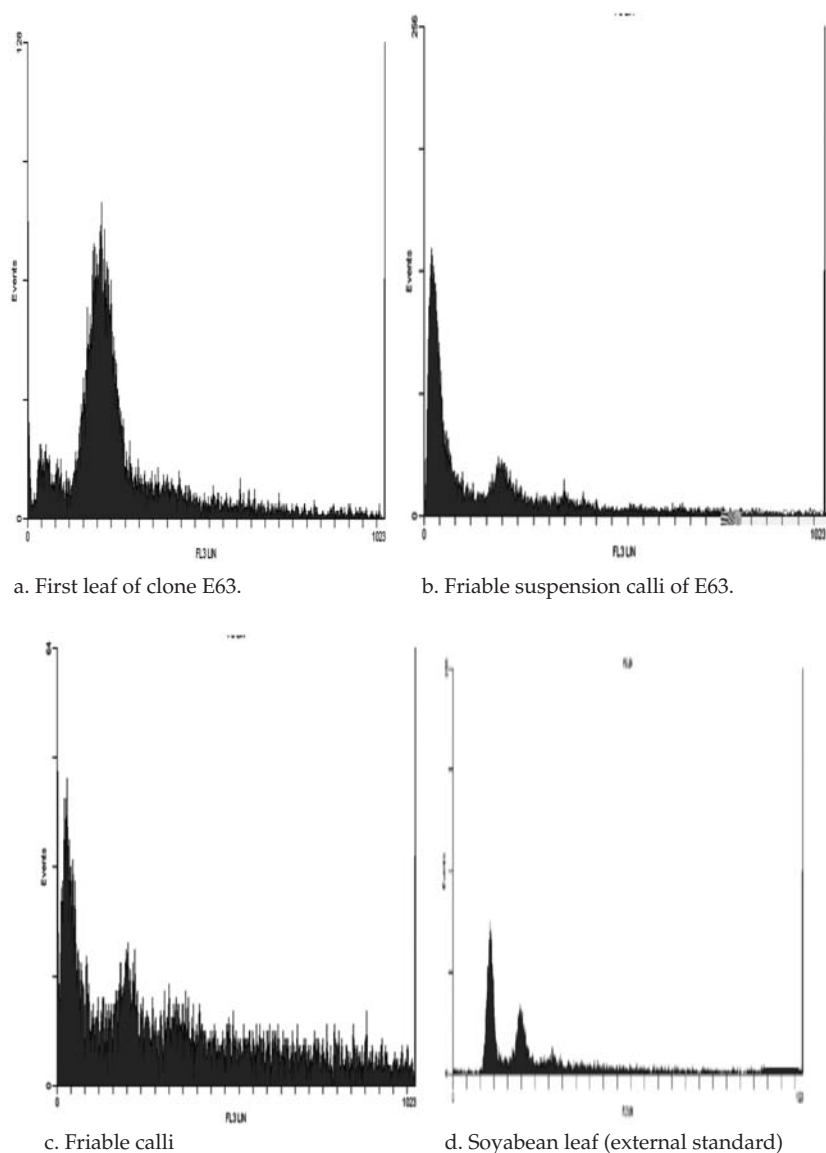
d. Soyabean leaf (external standard)

Figure 2. Fluorescent intensity histogram peaks of (a) clone E63 leaf, (b) E63 friable suspension calli, (c) friable calli and (d) soyabean standard.

Rooty and rooty suspension calli showed two IOD peak ranges, and this may be due to the heterogeneous structure of rooty calli (nodular calli with rooty structures). It may also be due to the 2C and 4C stages of nuclei activity; however, this was quite doubtful because the occurrence of the second IOD peak range did not double the value of the first IOD peak. The friable calli histogram chart show the IOD peak occurred at 220 000 units, and this is comparable to that of the nodular calli. The friable and nodular calli also showed the presence of nuclei having more than 500 000 units IOD, demonstrating the presence of nuclei with a high DNA content. The root tips of clones E63 and E85 showed IOD peaks at 120 000 and 140 000 units, correlating with the 2C DNA content of 3.29 pg and 4.04 pg, respectively. In general, all samples showed IOD peaks lower than the soyabean peak which was 220 000 units (2C = 2.5 pg) except for FSC E73-SG, FC, NC and RC, even though by FCM analysis, all samples contained more DNA than soyabean. This may be due either to the low number of cells analysed (100-200 nuclei) compared to the thousands of nuclei analysed by FCM, or to stoichiometric errors. Stoichiometric errors in dye binding caused by secondary plant metabolites such as polyphenols and low molecular weight substances such as catechin and quercetin may also play a role in reducing the intensity of Feulgen staining and influence fluorochrome binding (Greilhuber, 1988; 1997). However, stoichiometric errors are not present in fluorochrome binding of oil palm nuclei for FCM analysis (M Noirot, pers. comm., 2003). In this analysis, it was felt that the Feulgen staining of soyabean nuclei may have been influenced by stoichiometric errors due to the presence of many nuclei which had more than 500 000 IOD units (*Table 2*).
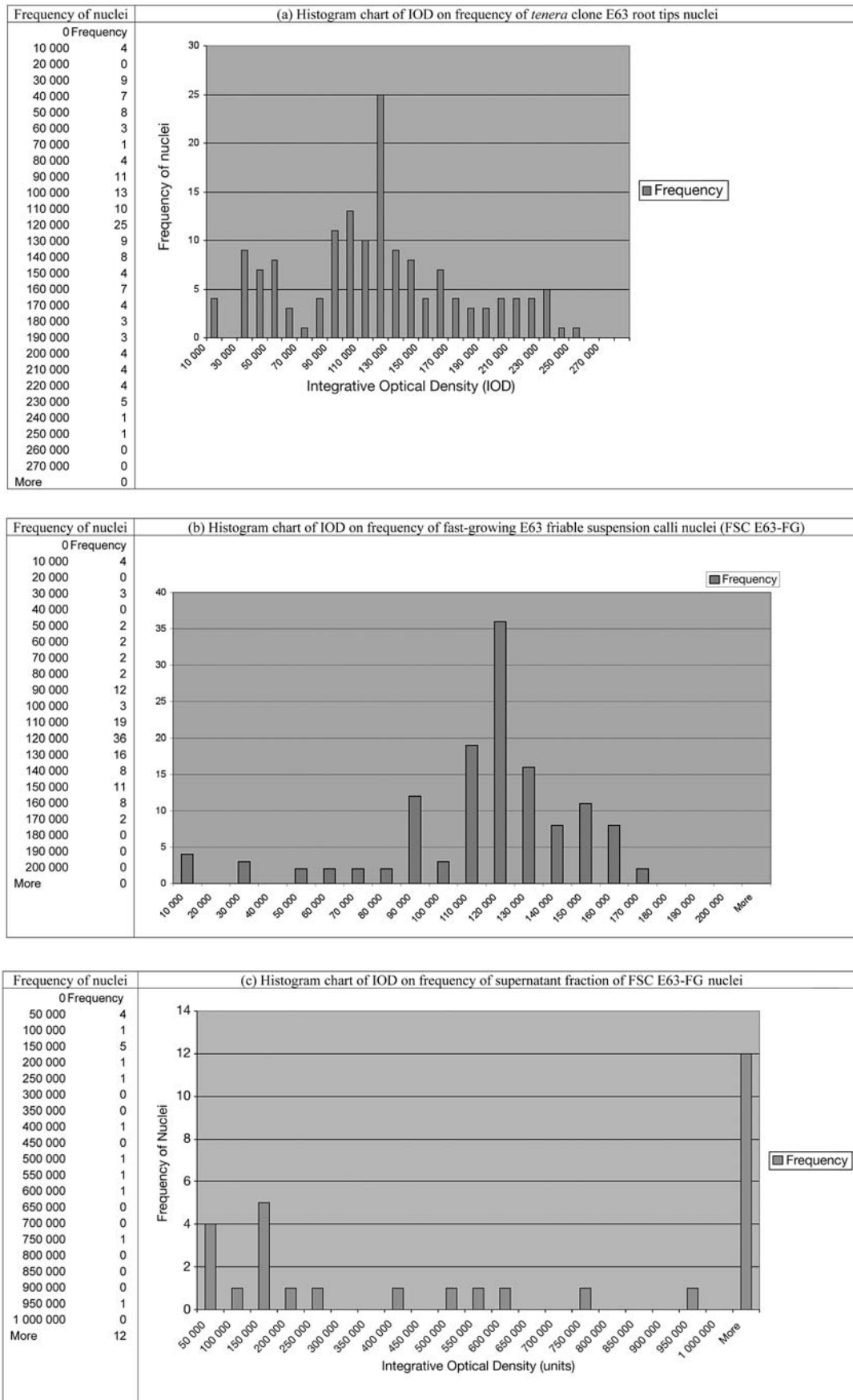
Frequency of nuclei	
	0 Frequency
10 000	4
20 000	0
30 000	9
40 000	7
50 000	8
60 000	3
70 000	1
80 000	4
90 000	11
100 000	13
110 000	10
120 000	25
130 000	9
140 000	8
150 000	4
160 000	7
170 000	4
180 000	3
190 000	3
200 000	4
210 000	4
220 000	4
230 000	5
240 000	1
250 000	1
260 000	0
270 000	0
More	0

(a) Histogram chart of IOD on frequency of *tenera* clone E63 root tips nuclei

Frequency of nuclei	
	0 Frequency
10 000	4
20 000	0
30 000	3
40 000	0
50 000	2
60 000	2
70 000	2
80 000	2
90 000	12
100 000	3
110 000	19
120 000	36
130 000	16
140 000	8
150 000	11
160 000	8
170 000	2
180 000	0
190 000	0
200 000	0
More	0

(b) Histogram chart of IOD on frequency of fast-growing E63 friable suspension calli nuclei (FSC E63-FG)

Frequency of nuclei	
	0 Frequency
50 000	4
100 000	1
150 000	5
200 000	1
250 000	1
300 000	0
350 000	0
400 000	1
450 000	0
500 000	1
550 000	1
600 000	1
650 000	0
700 000	0
750 000	1
800 000	0
850 000	0
900 000	0
950 000	1
1 000 000	0
More	12

(c) Histogram chart of IOD on frequency of supernatant fraction of FSC E63-FG nuclei

Figure 3. Histograms of integrative optical density (IOD) bins associated with the nuclei frequency of (a) tenera *clone E63 root tips, (b) E63 fast-growing friable suspension calli (FSC E63-FG), and (c) supernatant fraction of FSC E63-FG nuclei.*

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Fluorescence *in situ* Hybridisation of 18S-25S rDNA

FISH of rDNA was performed basically to establish a FISH method that is applicable to oil palm calli and also to determine their ploidy level cytologically. For example, an interphase diploid cell will show two major signals, while a triploid will exhibit three large signals, and so on and so forth. The FISH experiments were done on FSC, FC and NC, and it was observed that most interphase cells showed two large signals indicating the diploid state of cells (*Figure 4*). The large signals may be due to endoreduplication of DNA (Gilissen *et al.*, 1994; Shiba and Mii, 2005). Rival *et al.* (1997) and Jones *et al.* (1982) have also reported the diploid state of oil palm calli via FCM and microdensitometric analysis, respectively.

Reprogramming of Genome in Tissue Culture: Methylation, Replication, Chromatin Packaging

Demonstrations of hormonal control in the differentiation (Skoog and Miller, 1957) and totipotency of single plant cells (Steward *et al.*, 1958) laid the foundation for the clonal propagation or micropropagation of plants through tissue culture techniques (Kharkwal and Roy, 2004). The development of techniques and protocols to produce plant embryos asexually has had a huge technological and economic impact on agricultural systems, and currently these biotechnologies represent an integral part of the breeding programmes of agronomically important crops

(Maraschin *et al.*, 2005). Vegetative propagation of oil palm is not possible; hence, micropropagation is an extremely valuable strategy for multiplying elite lines, but propagation by tissue culture has led to somaclonal variations such as floral abnormalities (mantling) and have caused fruit abortion. At present, less than 2% of the planted oil palm crop is derived from tissue culture.

In the above analysis, variations in DNA content were observed, and may be attributed to changes in the chromosome number and structure that resulted from chromosomal rearrangement, breakage and lagging, endopolyploidization, meiosis-like division and non-disjunction. All these processes give rise not only to DNA variations but also result in mutations at different levels (Vázquez, 2001; Zhao *et al.*, 2005). Chromosome breakage and rearrangement can cause late replication of heterochromatin, insertion and excision (Lee and Philips, 1998). During micropropagation, stressful *in vitro* conditions may cause the genome to undergo abnormal reprogramming, and hence genome expression may be reset or may not follow the same orderly sequence that occurs under normal conditions (Jain, 2001). The plant regenerated in such stressful environments may have undergone DNA resetting or restructuring which can give rise to variability at genomic and/or morphological levels (Zhao *et al.*, 2005).

DNA methylation is the most common covalent modification of DNA found *in vivo*. It plays an integral role in the regulation of gene expression and is involved in transcriptional gene silencing, the regulation of transposons, nucleolar dominance
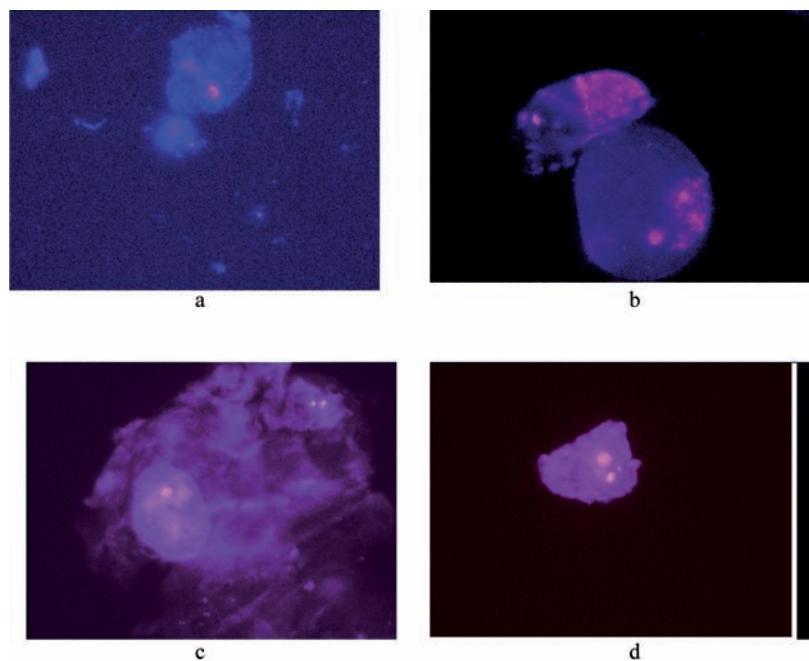

Figure 4. The rDNA sites in the interphase nuclei of (a) fast-growing friable suspension calli of E63 (FSC E63-FG), (b) supernatant fraction of FSC E63-FG, (c) friable calli, and (d) nodular calli. All images: 630X magnification.

and imprinting (Fieldes *et al.*, 2005). In each function, the methylated state is usually associated with inactivation of gene expression and, conversely, gene activation is associated with demethylation; moreover, DNA methylation influences the control and organisation of chromatin and its role in controlling gene expression (Fieldes *et al.*, 2005). For many plant species, including oil palm, the relationships between chromatin structure and gene expression and between histone-protein complexes, DNA methylation, and chromatin organisation are being unraveled. It has been reported that in oil palm, loss of methylation occurs during tissue culture (Jaligot *et al.*, 2000; 2001; Matthes *et al.*, 2001). Jaligot *et al.* (2004) reported that methylation deficit is associated with the mantled phenotype, and hence concluded the likelihood of a common regulatory pathway being affected in abnormal plants, while Tregear *et al.* (2002) suggested the existence of specific DNA sequences in the oil palm genome which show ultra-sensitivity to tissue culture-induced methylation changes. Gurevich *et al.* (2005) reported that the phenotype with low level fruit set of the 'Barhee' date palm is quite similar to the mantled phenotype of oil palm produced in tissue culture. The former is characterised by low levels of fruit set, formation of supernumery carpels, alleviation of symptoms, and reversions of many palms to the normal phenotypes. Preliminary molecular fingerprinting analysis of normal *vs.* abnormal 'Barhee' palms did not detect any genetic variance specific to the off-type, suggesting that an epigenetic mechanism expressed by an altered DNA methylation pattern may be responsible for the formation of the 'Barhee' off-type phenotype (Cohen *et al.*, 2004).

Practical Applications of Whole-genome Analysis/DNA Measurements Related to Tissue Culture

The practical application of whole genome analysis/DNA measurements related to tissue culture is mainly to assess clonal fidelity. Changes in DNA content are known to occur by endoreduplication in the explant tissues during *in vitro* culture (Shiba and Mii, 2005). In some species, ploidy variation in tissue culture may have originated from the cells involved in the original source explant undergoing polysomaty, where tissues are composed of cells with different ploidy levels (D'Amato, 1985). Assessment of ploidy variation by FCM analysis has been recognised as useful for maintaining those cell lines with high plant regeneration ability for protoplast isolation and micropropagation in lily (Supaibulwatana and Mii, 1998; Aziz *et al.*, 1999). As a change in ploidy level can cause somaclonal variation, FCM, ICM and FISH of rDNA tools can be used to observe

changes in DNA content, to view the nuclei activity, and to determine ploidy level, respectively.

Rival *et al.* (1997) reported that clonal oil palm plantlets originating from nodular compact callus (NCC) have been shown to exhibit the mantled variant phenotype at an average level of 5%, whereas this rate attained 100% in plantlets derived from fast-growing callus (FGC). They also found that the FCM analysis did not show any significant difference between NCC and FGC. In this study, the FCM analysis for the slow-growing friable suspension calli showed a significantly higher DNA content while the ICM analysis showed a broader pattern of nuclei activity compared to the fast-growing friable suspension calli. Throughout the analyses, all samples showed the diploid state. Unfortunately, the regenerants from these FSC had not reached a reproductive stage; hence, any somaclonal variation effects, in particular the mantling effects, could not be observed at that stage.

In conclusion, the tools developed in these analyses can be used to analyse accurately the DNA content, to characterise the pattern of nuclei activity, and to determine the ploidy level of materials prior to initiation of liquid suspension culture. In addition to making gross observations of the growth of friable suspension calli, the above-mentioned tools can also be used to analyse the suspension cultures prior to subculturing, and hence ensure the oil palm's clonal fidelity, as well as to improve the efficiency and robustness of liquid cultures.

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