ScienceAsia 31 (2005): **23-28**

The influence of physical conditions on embryo and protoplast culture in oil palm (*Elaeis guineensis* Jacq.)

Theera Srisawat and Kamnoon Kanchanapoom*

Department of Biology, Faculty of Science, Prince of Songkla University, Thailand.

* Corresponding author, E-mail: kamnoon.k@psu.ac.th

Received 7 Jul 2004
Accepted 18 Nov 2004

ABSTRACT: Shoots with well developed roots were produced when oil palm embryos (*Elaeis guineensis* Jacq.) were imbibed for 48 h and cultured in liquid Y3 (Eeuwens, 1978) medium. Embryos, which were imbibed for 48 h and cultured in Y3 liquid medium containing 8.3 µM picloram and 4.5 µM 2, 4-dichlorophenoxyacetic acid (2, 4-D) gave the highest callus induction. Cell nuclei were isolated from embryos, calli, cell suspensions, shoots and roots, stained with propidium iodide and analyzed using flow cytometry. Characterization of the DNA content revealed that most of imbibed oil palm embryos were in G2/M phase of the cell cycle. Protoplasts were isolated from these embryos, calli and shoots, and the high percentage of cell division was derived from protoplasts cultured in liquid thin layer method. Colonies were obtained in protoplasts derived from the agarose embedding method; however no further development was evidenced.

KEYWORDS: *Elaeis guineensis* Jacq, flow cytometry, imbibition, oil palm, protoplast culture.

INTRODUCTION

Oil palm is an arborescent monocotyledon and propagated by seeds, which usually result in wide variation in the field. Therefore it is necessary to develop vegetative propagation to produce large number of plants from elite palm. *In vitro* germination has been used to improve the germination rates of oil palm embryos.[1-3] Protoplast culture is an alternative method to improve oil palm involving somatic hybridization and gene transformation. However, protoplast of oil palm is known to be recalcitrant in culture that is reflected mainly in the slowness of *in vitro* morphogenesis.[4] It is generally known that genome size of *in vitro* culture can be altered through changes either in the chromosome number or in the ploidy level. This alteration can be accomplished by measuring cell nuclei DNA content by means of flow cytometry. Currently, flow cytometry offers a simple, rapid, accurate and convenient method for determining ploidy levels of DNA, assessment and analyzing the cell cycle.[5] In higher plants, cell growth and division is a cyclic process which consists of cellular events and nuclear events. Somatic cells having 2C DNA content are in either G0 phase (noncycling) or G1 phase (cycling). Cycling cells subsequently enter S phase, wherein the nuclear DNA content is doubled, and then G2 phase. Analysis of the nuclear DNA content via flow cytometry provides an accurate measurement of the proportions of the cells in the various phases of the cell cycle.[6]

A preliminary study showed that the imbibition of embryos was the important factor on embryo development and protoplast culture. Therefore the aims of this investigation are to improve the embryo germination, assay the variation of DNA content and develop the protoplast isolation and culture techniques.

MATERIALS AND METHODS

Plant Materials

Oil palm mature seeds variety *Tenera* were kindly provided by Horticultural Research Center, Surat Thani Province, Thailand. The epicarp and pulp were removed prior to removing the endocarp. The kernel-embedded zygotic embryos were first surface-disinfected with 70% alcohol for 1 min, soaked in 40% (v/v) commercial Clorox solution (containing 0.5 ml of Tween-20 emulsifier per 100-ml solution) for 1 h, and then rinsed at least three times with autoclaved distilled water. The kernels containing embryos were aseptically immersed in sterilized distilled water for 0, 24, 48 or 72 h. Intact embryos were excised from the kernels with a scalpel filled with a #11 blade and transfer to culture medium.

Seedling, Callus and Cell Suspension Initiation

Excised embryos were initially cultured on either liquid or solid Y3 medium [7] without plant growth regulators for seedling germination. The liquid Y3 media supplemented with 4.1-8.3 µM picloram, 4.5-13.6 µM 2, 4-D, 0.56 mM myo-inositol and 131 mM sucrose were used for callus induction. Callus initiated from embryos was observed for 8 weeks. Suspension cultures

were initiated by transferring 0.5 g of callus into a 125-ml flask containing 20 ml of liquid medium supplemented with 10.74 μM α-napthaleneacetic acid (NAA). The inoculum was placed on an orbital shaker with continuous shaking at 100 rpm.

Culture Media and Conditions

The pH of all media was adjusted to 5.7 prior to the addition of 0.82% Pearl Mermaid commercial gelling agent and autoclaved at 121°C for 20 min. All cultures were incubated at 25 ± 2°C air temperatures in a culture room with a 16 h photoperiod at 20 μmol $m^{-2}s^{-1}$ photosynthetic photon fluxes at plant level provided by cool-white fluorescence. Five embryos were planted per culture vessel. All experiments were carried out at least three times with five cultures per treatment.

Flow Cytometric Analysis

Ploidy level of embryos, calli, cell suspension, shoots and roots of oil palm were analyzed by measuring relative DNA content of isolated nuclei using a flow cytometer FACScalibur Becton Dickinson as previously described by Pfosser *et al*.[8] Briefly, plant materials (typically 20 mg) were chopped using a sharp razor blade in 1 ml of ice-cold Tris-$MgCl_2$ buffer in a glass Petri dish. This solution was filtered through a 42 μm nylon mesh, and subsequently 50 μl of 50 mg/ml propidium iodide and 50 μl of 50 mg/ml RNase were added immediately. The reference marker (*Raphanus sativus* L. cv. Saxa nova; 2C=1.11 pg) was kindly provided by Dr. Jaroslav Dolezel, Laboratory of Molecular Cytogenetics and Cytometry, Institute of Experimental Botany, Czech Republic.[9] Histograms of DNA contents were analyzed by WinMDI version 2.8.

Protoplast Isolation

For isolating protoplasts, imbibed-embryos, basal leaves and calli were preplasmolysed in a 5 ml of cell protoplast washing media (CPW)[10] salt solution and 0.7 M sorbitol for 1 h in the dark. After removing CPW solution, explants were chopped and treated with different concentrations of enzyme solutions containing Cellulase Onozuka R-10 (Yakult Honsha Co., Ltd., Japan Lot # 201059), Driselase (Kyowa Hakko Co., Ltd., Japan Lot # 4111) and Macerozyme R-10 (Yakult Honsha Co., Ltd., Japan Lot # 202021) dissolved in CPW salts and 0.6 M sorbitol at pH 5.7. Five ml of filter-sterilized enzyme solution were used for 0.2 g of each explant. The sliced embryos were incubated in the enzyme solutions (Table 1) without agitation for 24, 36, 48 or 72 h in darkness. Sliced shoots were incubated in 3.0% Cellulase Onozuka R-10, 1-2.5% Driselase and 1.0% Macerozyme-10, while calli were incubated in 1.0% Cellulase Onozuka R-10, 0.5% Driselase and 1.0% Macerozyme R-10 on an orbital shaker at 30 rpm for 20-48 h in the dark. After digestion, the cells and enzyme mixtures were screened through a 140 μm stainless steel sieve to remove undigested cells and debris. The digested cells were passed through two nylon sieve (77 μm followed by 42 μm pore size) and centrifuged at 52 g for 8 min. The pelleted protoplasts were then washed and purified by flotation on 0.6 M sucrose. 0.6 M sorbitol solution was layered over a sucrose solution and centrifuged at 52 g for 10 min. Protoplasts were collected from the interface between sucrose and sorbitol layers and resuspended in 0.5 M sorbitol.

Protoplast yield was determined using an AO Bright-Line hemacytometer slide at 100× magnification and their viability assessed using fluorescein diacetate (FDA).[11] The presence of wall material was monitored using Calcofluor white.[12] The fluorescence of the viable protoplasts was monitored using an Olympus inverted microscope model IMT-2 using a blue excitation filter (490-515 nm) and wall formation using ultraviolet excitation filter (365-420 nm) combination. A 35-mm camera (Olympus SC 35, Japan) was coupled to the microscope for purpose of color photography (Kodacolor ASA 200 film, USA).

Protoplast Culture

Washed protoplasts were plated at 1×10^5 protoplasts per ml and cultured by agarose bead (0.4% Agarose Type VII, Sigma, USA), overlaid with liquid thin layer and semi-solid medium (0.3% agarose) techniques. Agarose bead was surrounded by liquid medium with the same composition and renewed at a 2-week interval. All media contained MS medium[13] salts supplemented with 8.3 μM picloram, 4.5 μM 2, 4-D, 8.8 μM N^6-benzyladenine (BA) and 4.6 μM kinetin. Protoplasts were kept in the dark in a constant temperature room at 25 ± 2°C. When cell division was evidenced, the osmotic pressure of the medium was gradually reduced at a 14-day interval.

Data Analysis

All experiments were repeated twice with three replicates per treatment. Four to five protoplast samples were taken randomly from each treatment for the determination of their means and standard errors (SE). The frequency of cell division was calculated after 2 weeks of culture and was statistically analyzed by analysis of variance (ANOVA) at $p \leq 0.05$.

Results

When intact zygotic embryos were cultured either on solid or in liquid Y3 media without plant growth regulators, enlargement of the embryos was clearly visible and they underwent germination. Embryos were recorded as viable when the first leaf emerged. Seedlings

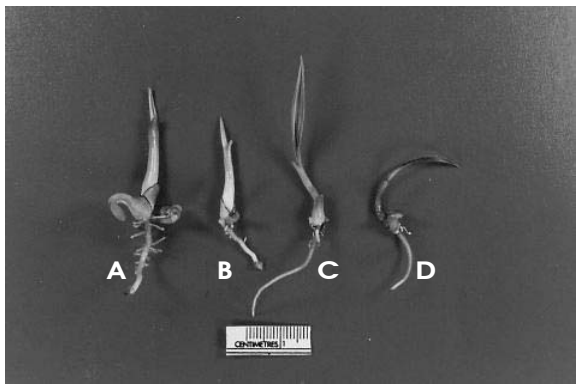

Fig 1. Germination oil palm seedlings from imbibed (A and C) and non-imbibed (B and D) embryos cultured in liquid (A and B) or on solid (C and D) Y3 media without plant growth regulators after cultured for 28 days.

usually showed normal geotropic responses, growth and development in terms of length and weight. The roots of seedlings were single and primary roots whereas the secondary roots developed from these. The growth of the embryos derived from imbibed embryos was achieved better than unimbibed embryos. Imbibition resulted in improved germination and produced vigorous plantlets (Fig. 1a, c), while unimbibed embryos showed abnormal growth and development (Fig. 1b, d). Since imbibition was effective in causing germination, experiments were conducted to imbibe embryos for varying periods of times. A correlation was observed between the duration of imbibition and growth of embryos. The results revealed that the growth of embryos varied greatly with different imbibition times. Fresh weight and length of shoot and root of seedlings in embryos imbibed for 48 h measured during the imbibition period. Shorter or longer period of imbibition did not increase the germination percentage significantly. It was found that the fresh weight of 48 h-imbibed embryos was 1.647 g ($p \leq 0.0001$), while the lengths of shoot and root were 8.6 cm ($p \leq 0.001$) and 6.8 cm ($p \leq 0.001$), respectively, after being cultured for 8 weeks (Fig. 2).

Callus induction and embryo germination were obtained when both unimbibed and imbibed embryos at different times were cultured in liquid Y3 media with the addition of picloram and 2, 4-D during the first two months in culture. Picloram, a herbicide with auxin-like properties, was found to be as effective as 2, 4-D. From preliminary results, 13.6 µM 2, 4-D was superior in callus formation, hence picloram was used in combination with 2, 4-D. There were no significant differences among media treatments (Fig. 3); however, Y3 liquid medium containing 8.3 µM picloram and 4.5 µM 2, 4-D gave the highest callus weight. The texture of the callus was friable and callus formation varied with the imbibition times significantly ($p \leq 0.001$) at the 95% confidence level. The percentage of embryos forming callus was higher in imbibed embryos than unimbibed embryos. The highest callus weight observed on imbibed embryos for 48 h was 161.01 mg per embryo. Therefore this 48 h period was used for routine imbibition.

Flow cytometry was performed to measure DNA content of imbibed embryos. The standard peak of 2C and 4C of *Raphanus sativus* L. cv. Saxa nova leaves was determined on each measuring date. The peak positions did not show any variation and the genome size of oil palm was estimated as 2C=3.67 pg (Fig. 4). To determine

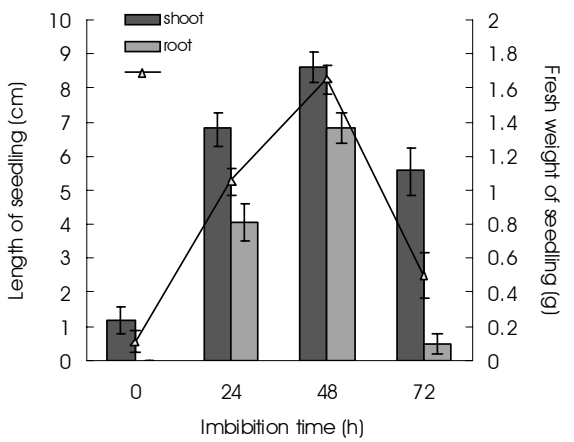

Fig 2. Effect of imbibition times on fresh weight, shoot and root length of oil palm seedlings derived from imbibed and non-imbibed embryos cultured in Y3 liquid medium after cultured for 56 days. Error bars represent ±SE.

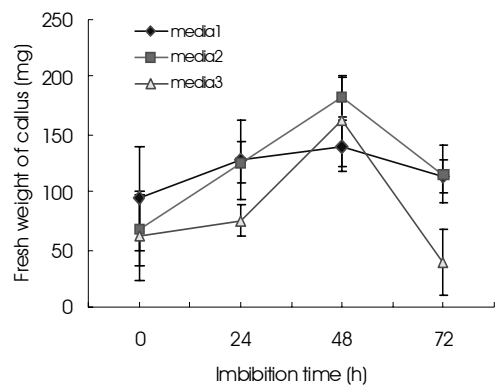

Fig 3. Callus fresh weight derived from imbibed embryos for 0, 24, 48 or 72 hours cultured in Y3 liquid medium supplemented with 4.1 µM Picloram and 9.0 µM 2, 4-D (media 1); 8.3 µM Picloram and 4.5 µM 2, 4-D (media 2) and 13.6 µM 2, 4-D (media 3) after cultured for 56 days. Error bars represent ±SE.

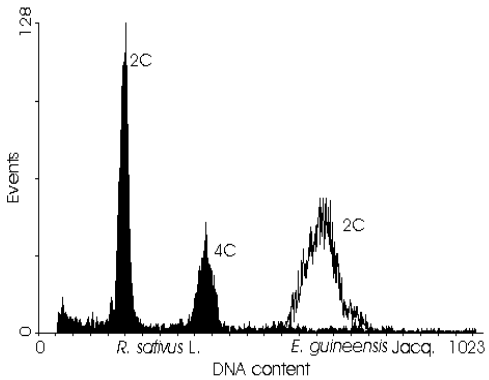

Fig 4. Flow cytometric analysis of the DNA content of *Elaeis* embryos using *Raphanus* leaves as a reference marker.

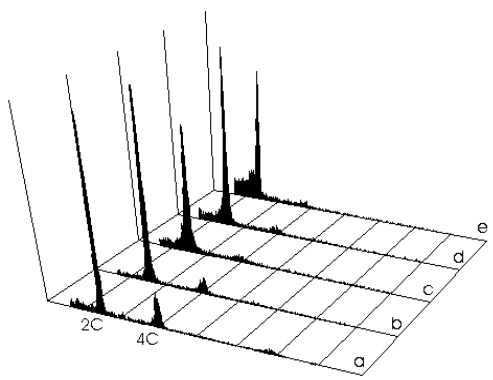

Fig 5. Histogram of propidium iodide fluorescence intensity of oil palm nuclei isolated from imbibed embryos for 48 hour (a), calli (b), cell suspension (c), shoots (d) and roots (e) . The fluorescence histograms were resolved into 2C and 4C cell cycle compartments with a peak-reflect algorithm using Win MDI version 2.8. X-axis represents DNA content; Y-axis represents event of cell counts.

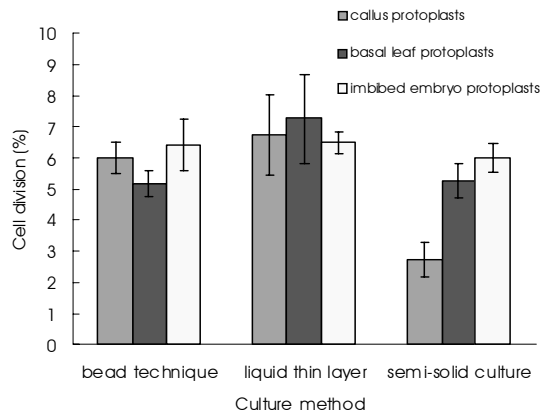

Fig 6. Percentage of cell division of oil palm protoplasts from different sources of explants cultured on MS medium using agarose as gelling agent supplemented with 8.3 µM picloram, 4.5 µM 2, 4-D, 8.8 µM BA and 4.6 µM kinetin after cultured for 7 days. Error bars represent ±SE.

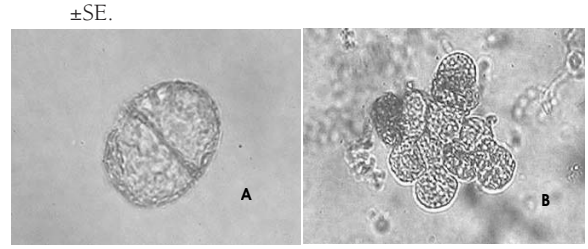

Fig 7. (A) Dividing protoplast in the medium containing 8.3 µM picloram, 4.5 µM 2, 4-D, 8.8 µM BA and 4.6 µM kinetin 2-3 days after culture. (B) Cell colony formation of oil palm protoplasts cultured in agarose bead MS medium containing 4.5 µM dicamba, 4.5 µM 2, 4-D, 9.3 µM kinetin, 4.4 µM BA and 3.8 µM ABA (Abscisic acid) after cultured for 14 days.

the ploidy level, embryos, calli, cell suspension, shoots and roots of oil palm were analyzed for relative DNA content (Fig. 5). The distribution of the nuclei extracted from embryo imbibed for 48 h displayed a prominent peak at 4C indicating that they consisted of cells with G2/M phase of cell cycle (Fig. 5a). This profile is contrast to what was observed for calli, cell suspension, shoots and roots (Fig. 5b-e).

By 24 to 72 h of enzyme treatment, 0.35 to 4.52×10^6 protoplasts were routinely obtained per 0.2 gram fresh weight of 48 h imbibed embryos, and they showed 30-80% viability as assessed by FDA staining (Table 1). No

Table 1. Yield (10^6 protoplasts $gram^{-1}$ fresh weight) and viability (%) of oil palm protoplasts isolated from imbibed embryos in various incubation times at 24, 36, 48 and 72 h with different enzyme mixtures.

%Enzyme mixtures (w/v)			Incubation time (hour)							
			24		36		48		72	
Cel[1]	Dri[2]	Mac[3]	Yield (×10^6 pp g^{-1}FW)	Viable (%)	Yield (×10^6 pp g^{-1}FW)	Viable (%)	Yield (×10^6 pp g^{-1}FW)	Viable (%)	Yield (×10^6 pp g^{-1}FW)	Viable (%)
1	0.25	0.25	1.49	68.65	1.75	37.43	4.52	38.48	4.02	40.80
1	0.25	0.50	1.15	48.20	1.85	53.34	1.00	51.87	1.25	82.80
1	0.50	0.25	0.39	38.14	1.97	59.48	1.25	49.30	0.35	57.86
1	0.50	0.50	1.91	56.83	1.03	30.75	1.54	77.51	1.46	39.05

Cel[1]: Cellulase Onozuka R10; Dri[2]: Driselase; Mac[3]: Macerozyme R10

protoplast isolation was obtained from root and cell suspension. Translucent protoplasts could be isolated from leaves whereas protoplasts from callus were relatively rich in cytoplasm (data not shown). In all media, protoplasts exhibited regeneration of a complete cell wall 24 h after the initiation of protoplast culture, with first mitotic division commencing 24 h later (Fig. 7A). Methods of protoplast culture significantly affected the relative percentage of cell division ($p \leq 0.05$). A high percentage of division was obtained when protoplasts were cultured either in liquid thin layer at a frequency of 6.91±0.54% or agarose bead at a frequency of 5.86±0.33%. The percentage of cell division obtained from semi-solid culture was significantly the lowest (4.66±0.50%). The effects of types of protoplast sources had no significant influences on subsequent cell division (Fig. 6). No difference was detected for imbibed embryo-derived protoplasts in all three culture methods whilst callus-derived protoplasts and leaf-derived protoplasts yielded inconsistency. Cell division was not sustained in cultures initiated from liquid thin layer or semi-solid technique. Continued cell divisions were only seen in protoplast cultures derived from the agarose embedding procedure. Subsequently, the protoplasts underwent further divisions and formed micro-colonies in the agarose-embedded culture medium, but no evidence of microcalli was seen (Fig. 7B).

Discussion

The physiological status of *in vitro* donor material is an important factor when cells or tissues are converted to protoplasts. Treatment of embryos prior to enzymatic digestion plays an important role in protoplast isolation and culture. As we have shown, an imbibition period, typically 48 h prior to culture of seeds, induces callus, and incubation in enzyme improves the efficiency of seedling germination, callus formation and protoplast isolation. Extended imbibition in water presents two potential problems, namely the possibility of excessive water uptake which could cause damage and subsequent leaching or, the possibility of water-logging of intercellular air spaces with the concomitant lowering of oxygen supply to embryo.[14] In oil palm, Rabechault *et al.*[15] reported that embryos developed successfully *in vitro* if the moisture content of the seeds was raised to 20-22% prior to removal of the embryos.

Determining ploidy levels in oil palm by counting chromosomes is difficult and time consuming. Oil palm chromosomes are small with the chromosome number of 32 and most often clumped together thus complicating the counting. To overcome these difficulties, flow cytometry was applied to determine the ploidy levels of oil palm. Flow cytometry has advantages over conventional chromosome counting since the method is simple, rapid, sensitive and convenient.[16] The flow cytometric analysis presented in this study suggests that the peak detected in the 4C position represent cells in the G2/M phase of the cell cycle. On this basis imbibed embryos for 48 h appear to be more efficient material for cell division since more cells were in G2/M phase than other imbibition times and will be considered as an initial material. Early germination is dominated by cell elongation, and enlargement with high DNA content may be advantageous during germination.[17] Mature oil palm seeds have embryos with the highest DNA content, hence growth may be accelerated by employing a strategy of increased rate of cell division.

In this study, imbibed embryos provided a large number of protoplasts which was greater than protoplasts from calli and seedlings (data not shown). We have demonstrated that a single enzyme combination provided a general method that gives a workable yield of viable protoplasts. Embryonic cells have large nuclei with dense cytoplasm and have several advantages including continuous accessibility to sterile plant material. For recalcitrant species such as oil palm, a high initial plating density (more than 10^5 protoplast per ml) is required. This is typically the case of woody plant species protoplasts.[18] Cell division was seen in protoplast cultures derived from thin liquid layer procedure. It is generally accepted that a gradual reduction of osmotic pressure supports sustained growth of newly protoplast-derived cell colonies; however the changing of the liquid medium surrounding the agarose beads in our experiment did not give positive results. Protoplast culture of oil palm has been reported from cell culture[19] and polyembryogenic culture.[20] In most cases only microcalli were produced, while in our study, microcalli were not able to develop further after they reached 5-10 cells (Fig. 7B). This is an important step since Ochatt *et al* [21] stated that for many protoplast systems particularly for woody plants, the ten-cell colony stage is the developmental threshold beyond which growth can be arrested.

In conclusion, this research has shown that 48 h imbibed embryos can be used as the source of embryo and protoplast culture. The development of a protoplast-to-tree system in oil palm, which is recalcitrant to protoplast culture, was achieved with this preliminary investigation, although a more thorough study of the nutritive conditions is needed to overcome the decline in growth of microcalli at early stage. Research is in progress on this aspect. Furthermore, flow cytometry can be used for the precise measurement of the nuclear DNA content in oil palm. The application of flow cytometry to estimate the

genomic constitution in other plant species is currently under way.

Acknowledgements

This paper represents a portion of Theera Srisawat's dissertation presented to the Graduate School, Prince of Songkla University. Research assistance to Theera Srisawat from the Thailand Research Fund through the Royal Golden Jubilee Ph.D. Program (Grant No. PHD 4BPS42B1) is acknowledged. This work was also financially supported by Prince of Songkla University's annual budget.

References

1. Alang Z and Fadzillah H (1986) A simple technique for embryo culture of oil palm (*Elaeis guineensis* Jacq.). *Report Special Research Project Tropical Agricultural Resources* **5**, 123-7.
2. Teixeira JB, Sondahl MR, Nakamura T and Kirby EG (1995) Establishment of oil palm cell suspensions and plant regeneration. *Plant Cell Tiss Org Cult* **40**, 105-11.
3. Chourykaew B and Kanchanapoom K (1996) In vitro culture of embryos and callus of oil palm (*Elaeis guineensis* Jacq.). *J Sci Soc Thailand* **22,** 1-12.
4. Sandoval A, Hocher V and Verdeil JL (2003) Flow cytometric analysis of the cell cycle in different coconut palm (*Cocos nucifera* L.) tissues cultured *in vitro*. *Plant Cell Rep* **22,** 25-31.
5. Winkelmann T, Sangwan RS and Schwenkle HG (1998) Flow cytometric analyses in embryogenic and non-embryogenic callus lines of *Cyclamen persicum* Mill.: relation between ploidy level and competence for somatic embryogenesis. *Plant Cell Rep* **17**, 400-4.
6. Dolezel J (1991) Flow cytometric analysis of nuclear DNA content in higher plants. *Phytochem Anal* **2**, 143-54.
7. Eeuwens CJ (1976) Mineral requirements for growth and callus initiation of tissue explants excised from mature coconut palm (*Cocos nucifera*) and cultured *in vitro*. *Physiol Plant* **36**, 23-8.
8. Pfosser A, Amon A, Lelley T and Heberle-bors E (1995) Evaluation of sensitivity of flow cytometry in detecting aneuploidy in wheat using disomic and ditelosomic wheat-rye addition lines. *Cytometry* **21**, 387-93.
9. Dolezel J, Sgorbati S and Sergio L (1992) Comparison of three DNA fluorochromes for flow cytometric estimation of nuclear DNA content in plants. *Physiol Plant* **85**, 625-31.
10. Power JB, Chapman JV and Wilson D (1984) Laboratory Manual, Plant Tissue Culture. Plant Genetic Manipulation Group. University of Nottingham, Nottingham, UK.
11. Widholm JM (1972) The use of fluorescein diacetate and phenosafranine for determining viability of cultured plant cells. *Stain Tech* **47**, 189-94.
12. Nagata T and Takebe I (1970) Cell wall regeneration and cell division in isolated tobacco mesophyll protoplasts. *Planta* **92**, 301-8.
13. Murashige T and Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol Plant* **15**, 473-97.
14. Nwankwo BN and Krikorian AD (1982) Water as a storage medium for *Elaeis guineensis* (pisifera) seeds under aseptic conditions. *Ann Bot* **50**, 793-8.
15. Rabechault H, Ahee J and Guenin G (1968) Researches sur la culture *in vitro* des embryons de palmier a huile *(Elaeis guineensis* Jacq) IV Effect de la teneur en eau des noix et de la duree de leur stockage. *Oleagineux* **23**, 233-7.
16. Dolezel J (1997) Application of flow cytometry for the study of plant genomes. *J Appl Genet* **38**, 285-302.
17. Price HJ, Morgan PW and Johnston JS (1998) Environmentally correlated variation in 2C nuclear DNA content measurements in *Helianthus annuus* L. *Ann Bot* **82**, 95-8.
18. Ortin-Parraga F and Burgos L (2003) Isolation and culture of mesophyll protoplast from apricot. *J Hortic Sci Biotech* **78**, 624-8.
19. Bass A and Hughes W (1984) Conditions for isolation and regeneration of viable protoplasts of oil palm (*Elaeis guineensis* Jacq.). *Plant Cell Rep* **3**, 169-71.
20. Sambanthamurthi R, Parman SH and Noor MRM (1996) Oil palm (*Elaeis guineensis* Jacq.) protoplast: isolation, culture and microcallus formation. *Plant Cell Tiss Org Cult* **46**, 35-41.
21. Ochatt SJ and Power JB (1989) Cell wall synthesis and salt (saline) sensitivity of colt cherry (*Prunus avium x pseudocerasus*) protoplasts. *Plant Cell Rep* **8**, 365-7.