4. RESULTS

4.1 Seedling and callus induction

4.1.1 Seedling induction

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 usually showed normal geotropic responses, growth and development in terms of length and weight. The seedlings produced single primary roots whereas the secondary roots developed from these. The embryos derived from imbibed embryos grew better than that of non-imbibed embryos, and showed improved germination and produced vigorous plantlets (Figure 6a, c), while non-imbibed embryos showed abnormal growth and development (Figure 6b, d). Since imbibition was effective in causing germination, experiments were conducted to imbibe embryos for various periods of time. A correlation was observed between the duration of imbibition and the growth of embryos. The results revealed that growth of embryos varied greatly with different imbibition times. Based on fresh weight and length of shoot and root of seedlings in embryos, 48 h was identified as the most suitable imbibition period. Shorter or longer periods of imbibition than 48 h did not increase the germination percentage significantly. It was found that fresh weight of 48h-imbibed embryos was 1.647 g ($p \leq 0.0001$) while length of shoot and root were 8.6

cm ($p \leq 0.001$) and 6.8 cm ($p \leq 0.001$), respectively after culture for 8 weeks (Figures 7 and 8).

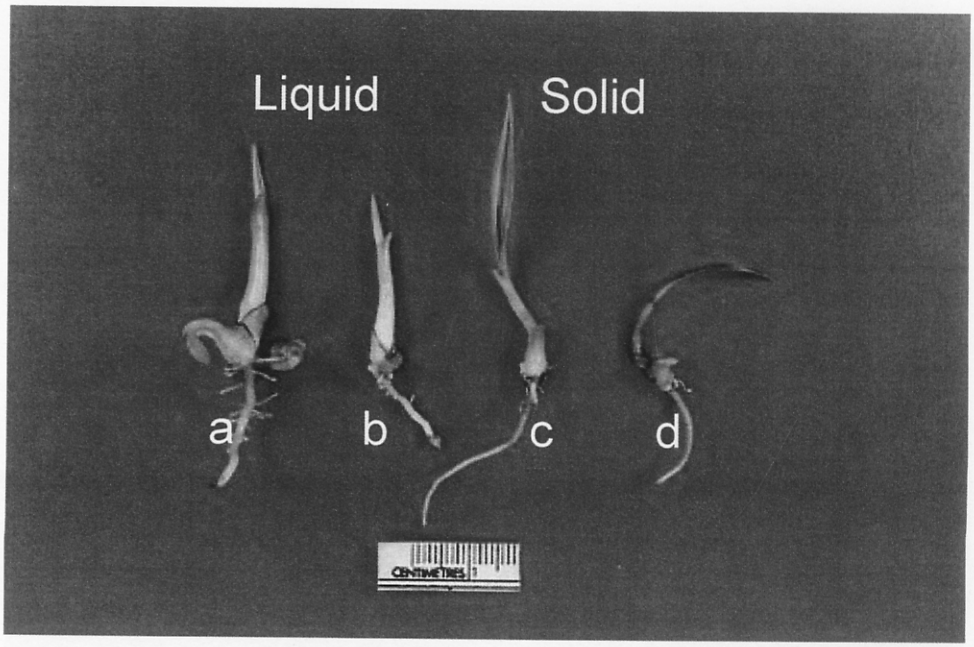


Figure 6 Germination oil palm seedlings from imbibed (a and c) and non-imbibed (b and d) embryos cultured in liquid (a and b) or on solid (c and d) Y3 media without plant growth regulators after culture for 28 days.

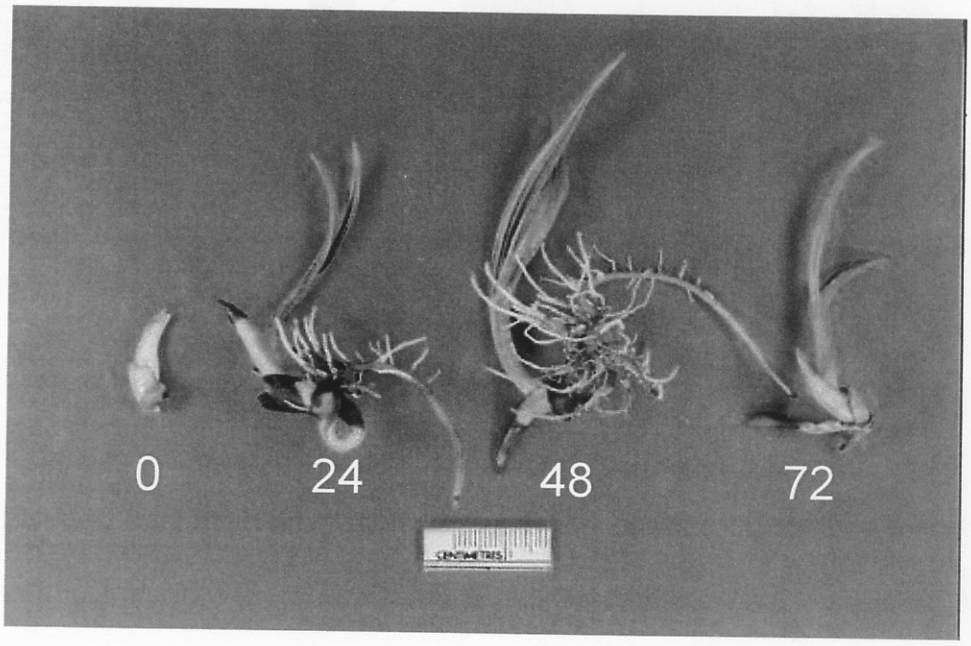


Figure 7 Germination oil palm seedlings from embryos imbibed for 24 48 and 72 h and non-imbibed (control) prior to cultured in liquid Y3 media without plant growth regulators after culture for 56 days.

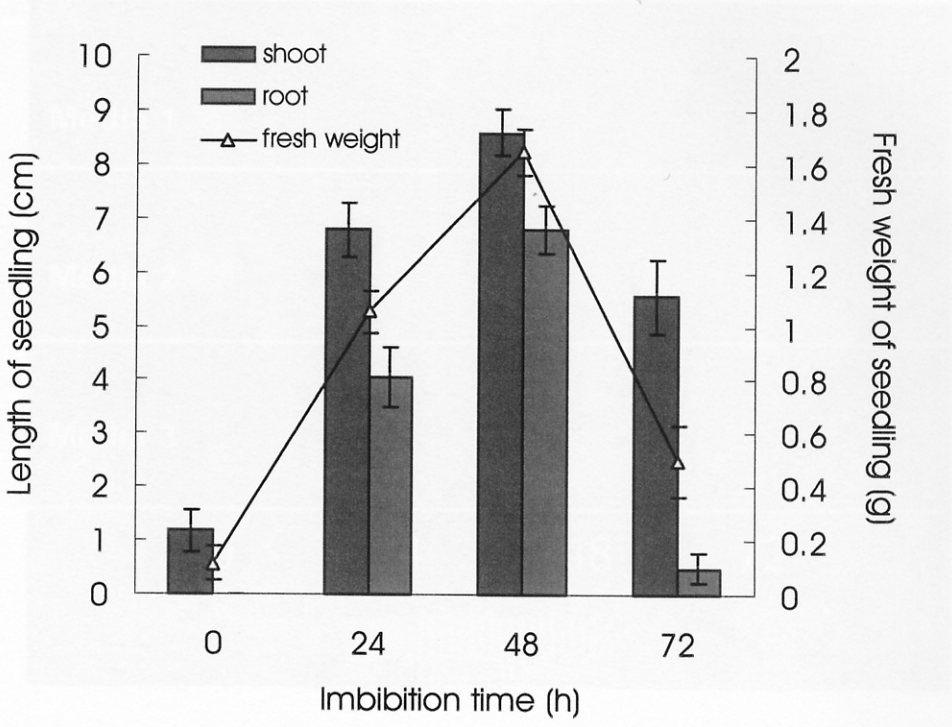


Figure 8 Effect of imbibition times on fresh weight, shoot and root length of oil palm seedlings derived from imbibed and non-imbibed embryos cultured in Y3 liquid medium after culture for 56 days. Error bars represent ±SE.

4.1.2 Callus formation

Embryos forming calli were obtained when both non-imbibed 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 (Figure 9). Picloram, a herbicide with auxin-like properties, was found to be as effective as 2,4-D and from preliminary results 13.6 μM 2,4-D was superior in callus formation, hence picloram was used in combination with 2,4-D.

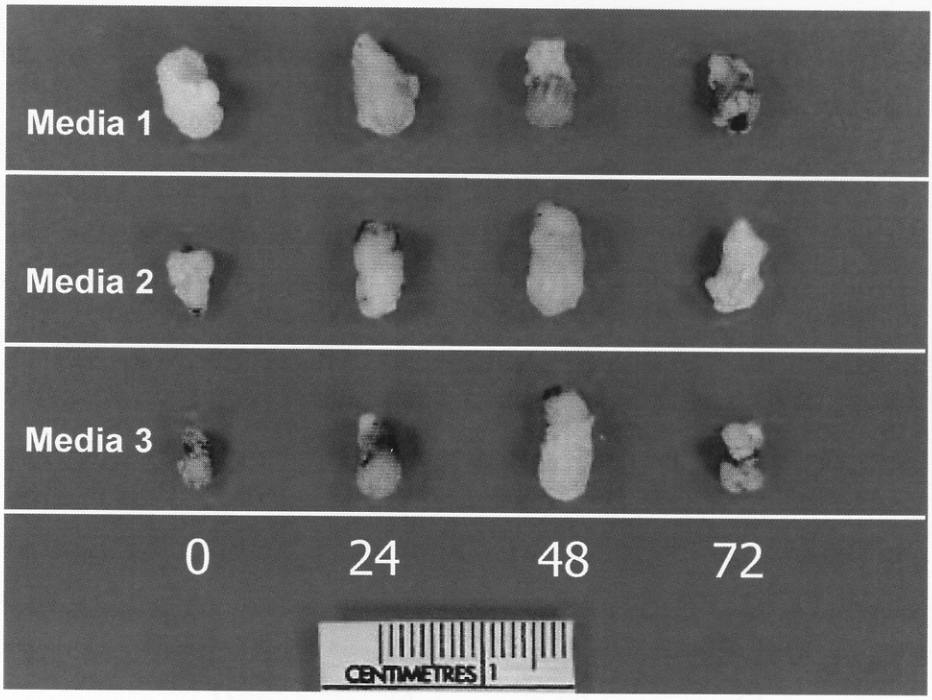


Figure 9 Callus formation from embryos imbibed for 0, 24, 48 and 72 hours prior to culture in liquid Y3 media supplemented with the following growth regulators, 4.1 μM picloram and 9.0 μM 2,4-D (media 1); 8.3 μM picloram and 4.5 μM 2,4-D (media 2) and 13.6 μM 2,4-D (media 3) after culture for 56 days.

Figure 10 shows that among the media treatments no significant differences were observed; 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 ($p \leq 0.001$). The percentage of embryos forming callus was higher in imbibed embryos than in non-imbibed

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.

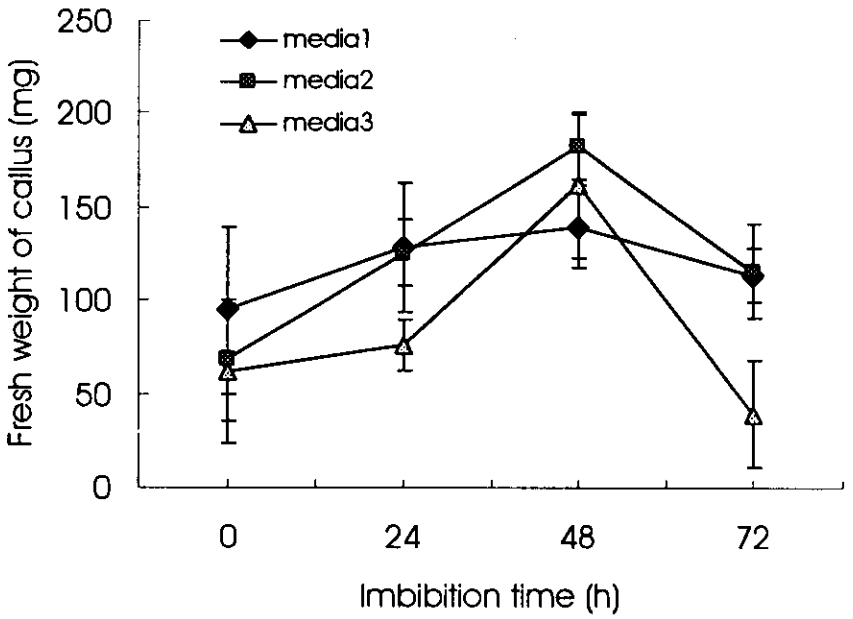


Figure 10 Fresh weight of calli derived from embryos imbibed for 0, 24, 48 or 72 hours after culture in Y3 liquid media supplemented with 4.1 μM picloram and 9.0 μM 2,4-D (media 1); 8.3 μM picloram and 4.5 μM 2,4-D (media 2) and 13.6 μM 2,4-D (media 3) after culture for 56 days. Error bars represent ±SE.

4.2 Flow cytometric analysis

4.2.1 Reference plants testing

DNA contents of oil palm cv. *Tenera* were analyzed using *Glycine max* cv. Polanka (2C=2.5; Afza *et al.*, 1993), *Lycopersicon esculentum* cv. Stupicke (2C=1.96; Dolezel *et al.*, 1992) and *Zea mays* line CE-777 (2C=5.72; Dolezel *et al.*, 1992) as

internal reference plants. DNA contents of oil palm were 4.18, 3.97and 4.34pg $2C^{-1}$ nuclei when *Glycine max* cv. Polanka, *Lycopersicon esculentum* cv. Stupicke and *Zea mays* line CE-777 were used as the reference plant, respectively. Thus the number of base pairs in one haploid genome was estimated in the range of $1.92\sim2.09\times10^9$ bp (Table 6 and Figure 11A-C). *Glycine max* cv. Polanka was used as an internal reference plant for routine DNA content analysis of oil palm on FACScalibur cytometer.

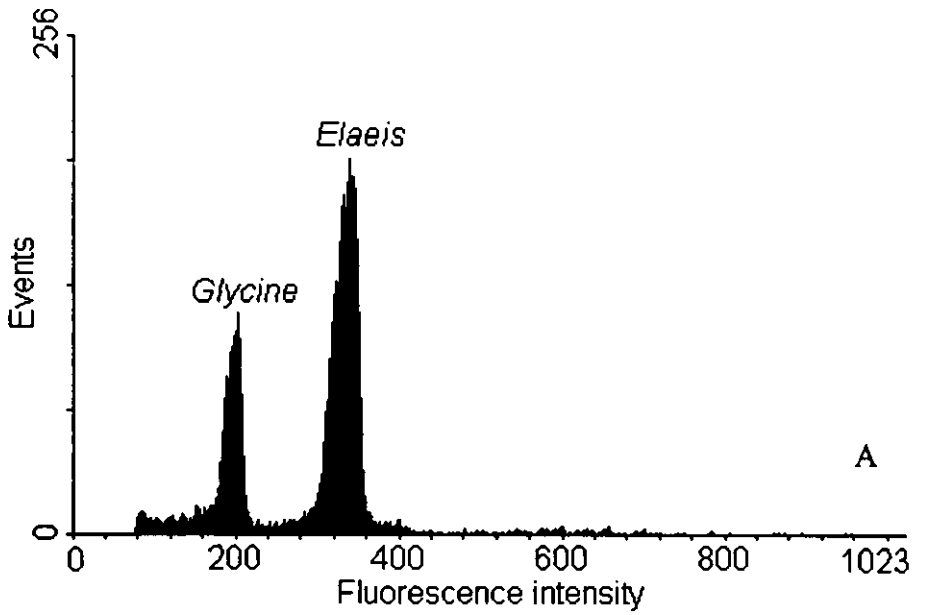
Table 6 Genome size (2C nuclear DNA content in pg) of *E. guineensis* Jacq. estimated by comparing with various reference plants. The means are based on 10 replicates per experiment.

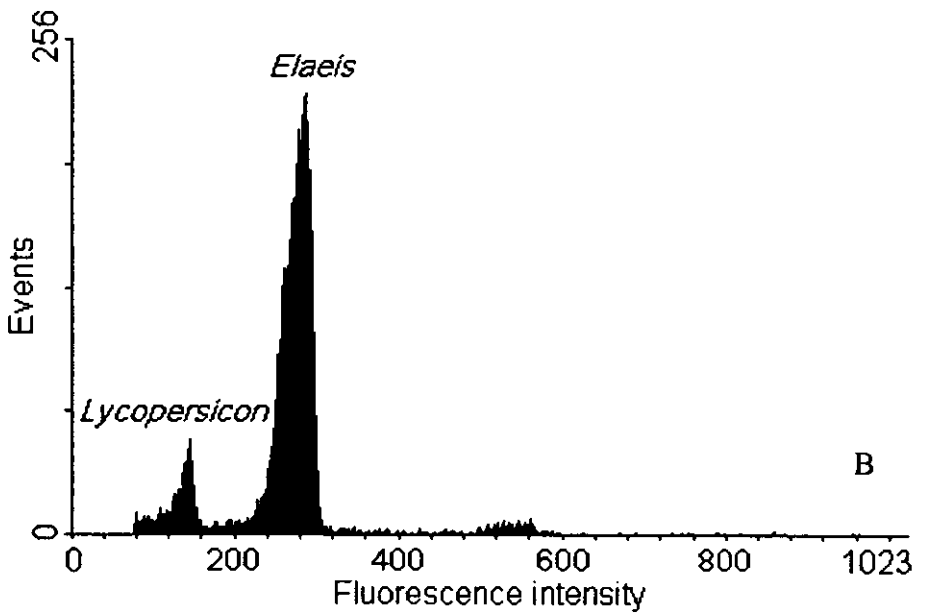
Reference plants	DNA content (pg $2C^{-1}$)		
	Reference plants	*E. guineensis* Jacq. (Mean±SE)*	*Elaeis* (Base pairs)[3]
G. max cv. Polanka	2.5[2]	4.18±0.04[b]	2.01×10^9
L. esculentum cv. Stupicke	1.96[1]	3.97±0.09[c]	1.92×10^9
Z. mays line CE-777	5.72[1]	4.34±0.03[a]	2.09×10^9

[1]Dolezel *et al.* (1992); [2]Afza *et al.* (1993)

[3]Base pairs per haploid genome (Bp $1C^{-1}$ nuclei) calculated based on the equivalent of 1 pg DNA=965 Mega bp.

*Values having superscripts in common are not significantly different at the 0.05 probability level by Tukey test.


256
Events
0
Elaeis
Glycine
A
0
200
400
600
800
1023
Fluorescence intensity


256
Events
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Elaeis
Lycopersicon
B
0
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1023
Fluorescence intensity

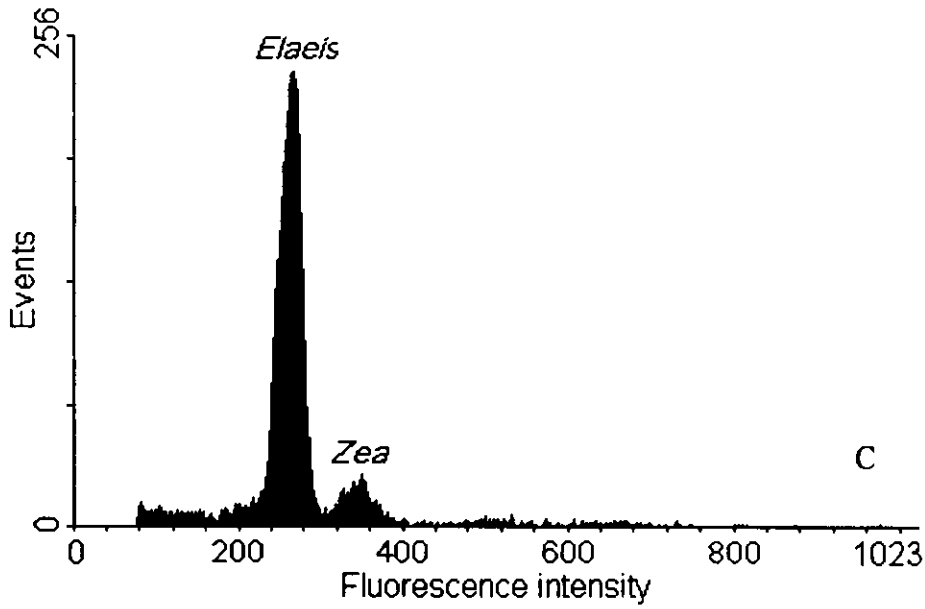


Figure 11 Histogram of fluorescence intensity of nuclei isolated from oil palm using *Glycine max* cv. Polanka (A), *Lycopersicon esculentum* cv. Stupicke (B) and *Zea mays* line CE-777 (C) as internal reference plants.

4.2.2 Genomic DNA alteration

To investigate the change in DNA content of oil palm tissues cultured *in vitro* for one year, *Glycine max* cv. Polanka was used as an internal reference. Histogram of relative DNA content histograms and DNA contents of cultured oil palm tissues obtained in these experiments are shown in Figure 12A-C and listed in Table 7, respectively. The results revealed that embryos and one-year-old shoot

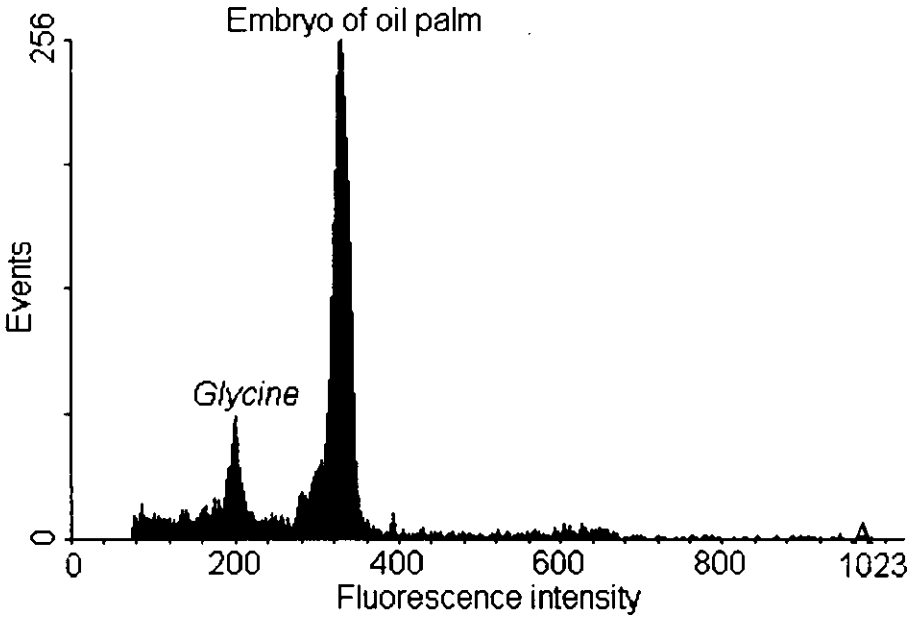
meristems of seedlings at the third subculture in liquid MS medium without growth regulator were significantly different in their DNA contents from those of calli at the third subculture on MS medium containing 4.1-41.4 μM picloram or 4.5-45.2 μM dicamba (4.16 in embryo, 4.15 in shoot meristem and 4.68 pg $2C^{-1}$ in calli, respectively, $p \leq 0.0001$).

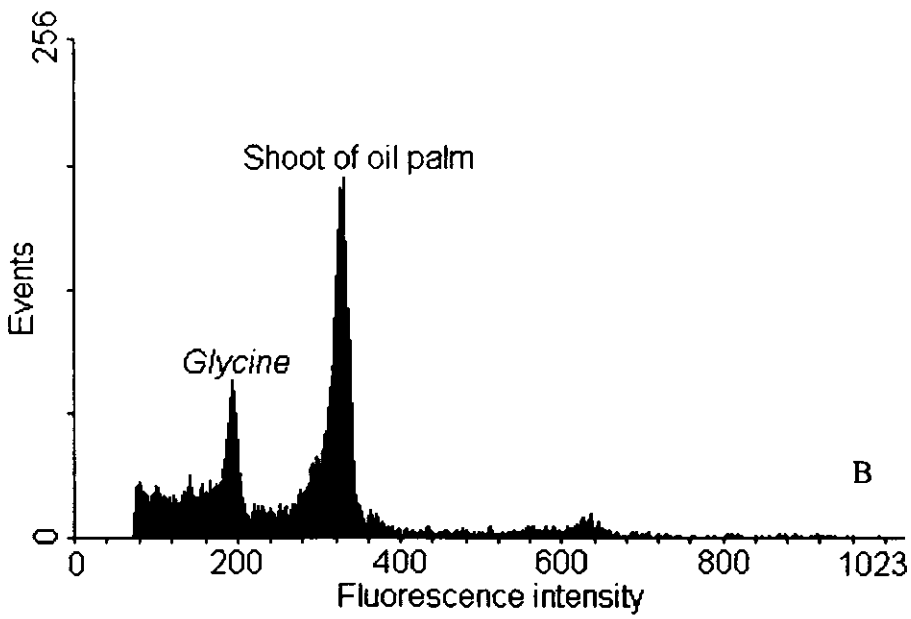
Table 7 Genome size (2C nuclear DNA content in pg) of oil palm embryos, one-year-old shoot meristem of seedlings and calli at the 3rd *in vitro* subculture. The means are obtained from experiments with 5 replicates.

Explants of oil palm	DNA content (pg $2C^{-1}$) (Mean±SE)*
G. max cv. Polanka (internal reference)	2.5[1]
Embryos	4.16±0.02[b]
Shoots meristem	4.15±0.02[b]
Calli	4.68±0.03[a]

[1]Afza *et al.* (1993)

*Values having superscripts in common are not significantly different at the 0.05 probability level by Tukey test.


Embryo of oil palm
256
Events
0
Glycine
A
0
200
400
600
800
1023
Fluorescence intensity


256
Shoot of oil palm
Events
0
Glycine
B
0
200
400
600
800
1023
Fluorescence intensity

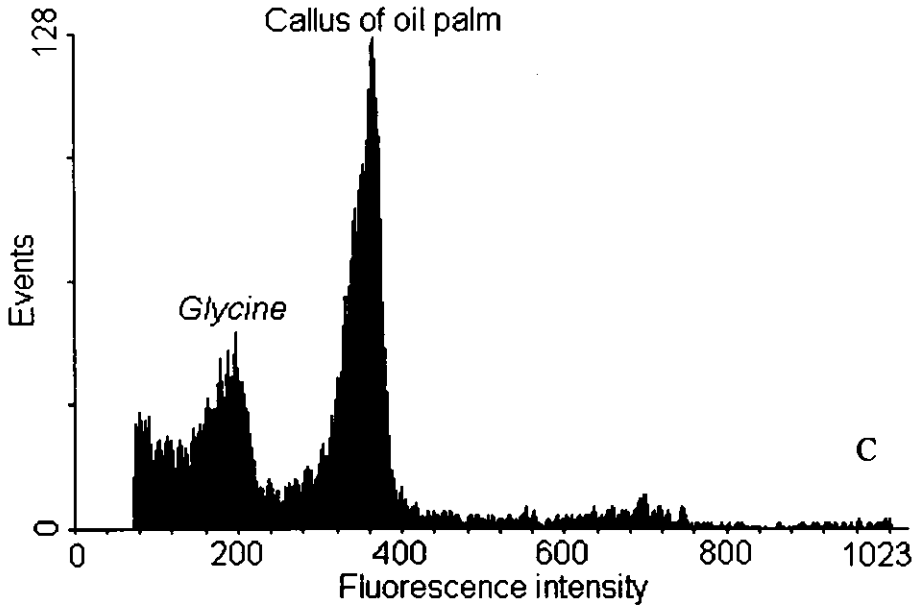


Figure 12 Comparison of fluorescence intensity in nuclei of oil palm among embryos (A), long-term *in vitro* cultured tissues of shoot meristem (B) and calli (C) using *Glycine max* cv. Polanka as an internal reference.

4.2.3 Cell cycle activity analysis

The standard peaks of 2C and 4C of reference leaves were determined on each measuring date. To determine the proportions of cells in the G2 phase of the cell cycle, embryos, calli, shoots and roots of oil palm were analyzed for cell cycle activity (Figures 13 and 14). Figure 13 shows the distribution of the cell population of embryos imbibed for 24, 48 and 72 h. The distribution of the nuclei extracted from embryos imbibed for 48 h displayed a prominent peak at 4C (4.53%), indicating that

cells with higher proportion of cells with G2/M phase of cell cycle (Figure 13 and 14a). This profile is in contrast to what was observed for non-imbibed embryos and embryos imbibed for 24 and 72 h, calli, shoots and roots (Figures 13 and 14b-d).

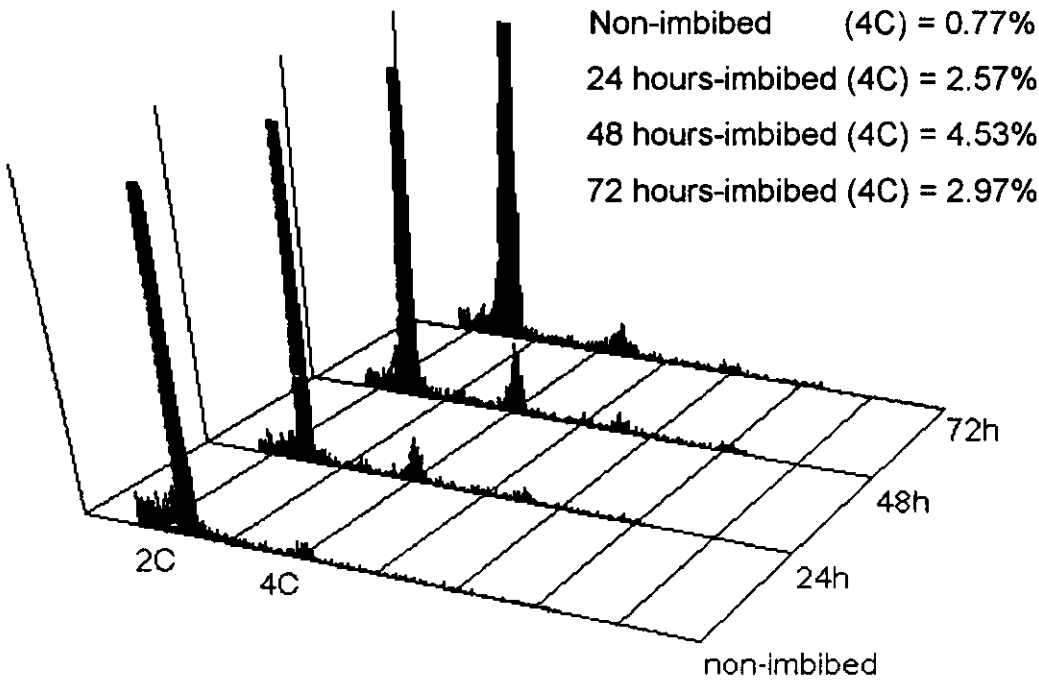


Figure 13 Histogram of propidium iodide fluorescence intensity of oil palm nuclei isolated from embryos imbibed for 24, 48 and 72 hour and non-imbibed embryos. The fluorescence histograms were resolved into 2C (G0/G1) and 4C (G2/M) cell cycle compartments with a peak-reflect algorithm using Win MDI version 2.8. X-axis represents DNA content; Y-axis represents event of cell counts.

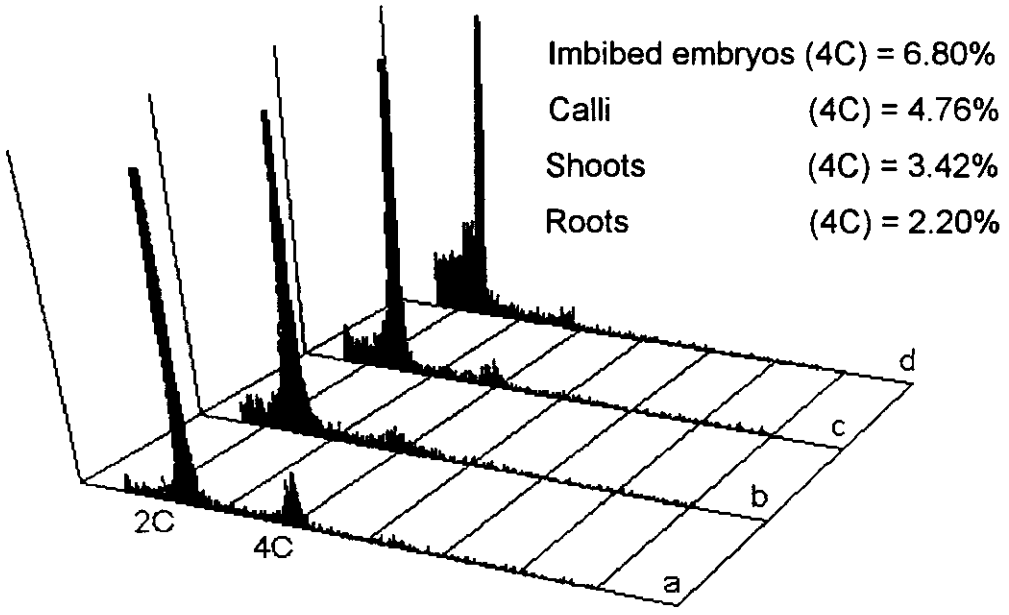


Figure 14 Histogram of propidium iodide fluorescence intensity of oil palm nuclei isolated from embryos imbibed for 48 hour (a), calli (b), shoots (c) and roots (d) . The fluorescence histograms were resolved into 2C (G0/G1) and 4C (G2/M) cell cycle compartments with a peak-reflect algorithm using Win MDI version 2.8. X-axis represents DNA content; Y-axis represents event of cell counts.

4.3 Protoplast isolation and culture

4.3.1 Protoplast isolation

4.3.1.1 Using seedlings and calli cultured on solid medium as protoplast sources

By 3 h of enzyme treatment, 5.53, 1.29 and 3.85×10^5 protoplasts were routinely obtained per 0.2 gram fresh weight of basal leaves, shoots and calli, respectively, using an enzyme consisting of 3% Cellulase Onozuka R-10 + 1.5% Driselase + 1% Macerozyme R-10 (Table 8). No protoplasts were isolated from root. Translucent protoplasts could be isolated from basal leaves whereas protoplasts from calli were relatively rich in cytoplasm (Figures 15A and B).

Table 8 Yield (10^5 protoplasts $gram^{-1}$ FW) of oil palm protoplasts isolated from seedlings and calli with different enzyme mixture after 3h of incubation.

Enzyme solution (%)			Yield (10^5 protoplasts $gram^{-1}$ FW)*		
Cellulase	Driselase	Macerozyme	basal leaf	shoot	callus
3	1	1	3.48 ± 1.86^{b}	1.08 ± 0.65^{ab}	3.02 ± 1.01^{b}
3	1.5	1	5.53 ± 1.26^{a}	1.29 ± 0.81^{a}	3.85 ± 1.31^{a}
3	2	1	2.12 ± 1.02^{c}	0.83 ± 0.42^{b}	3.82 ± 1.36^{a}
3	2.5	1	4.28 ± 1.25^{b}	1.08 ± 0.65^{ab}	1.38 ± 0.58^{c}

*Values having superscripts in common are not significantly different at the 0.05 probability level by Tukey test.

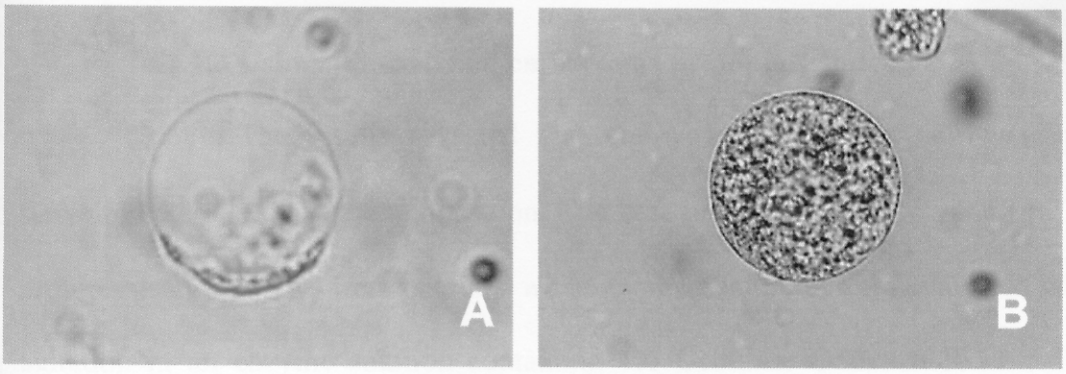


Figure 15 Freshly isolated protoplast from basal leaf (A) and callus (B).

4.3.1.2 Using friable calli cultured in liquid medium as protoplast source

The highest yield of protoplast from friable calli (1.77×10^6 protoplast $gram^{-1}$ FW) was obtained by using an enzyme solution consisting of 1% Cellulase + 0.5% Driselase + 1% Macerozyme and an incubation time for 24 h (Table 9).

Table 9 Yield of oil palm protoplasts isolated from friable callus cultured in liquid media with enzyme mixture of 1% Cellulase + 0.5% Driselase + 1% Macerozyme.

Incubation time (h)	Yield (10^6 protoplasts $gram^{-1}$ FW)*
20	0.49 ± 0.19^b
24	1.77 ± 0.95^a
28	0.69 ± 0.31^b
48	0.49 ± 0.23^b

*Values having superscripts in common are not significantly different at the 0.05 probability level by Tukey test.

4.3.1.3 Using 48h-imbibed embryos as protoplast source

The results revealed that embryos imbibed for 48h were to be suitable materials for enzyme digestion. The best results in terms of yield (4.77×10^6 protoplasts $gram^{-1}$ FW) were obtained when imbibed embryos were incubated without agitation in an enzyme solution containing 1% Cellulase Onozuka R-10 + 0.25% Driselase + 0.5% Macerozyme R-10 for 48 h (Table 10). Microscopic observation revealed that freshly isolated protoplasts from imbibed embryos were homogeneous in size (approximately 20-30 μm in diameter) with dense cytoplasm (Figure 16A). Viability of freshly isolated protoplasts was in the range of 30-80% by FDA staining (Figure 16B) and no fluorescence was observed when they were stained with Calcofluor White. Imbibed embryos provided clean and large number of protoplasts, which was higher than that of protoplasts from seedlings and calli (Tables 8, 9 and 10).

Table 10 Yield (10^6 protoplasts $gram^{-1}$ FW) and viability (%) of oil palm protoplasts isolated from 48h-imbibed embryos with different enzyme mixtures.

Enzyme mixtures (% w/v)			Incubation time (hour) without agitation							
			24		36		48		72	
[1]Cel	[2]Dri	[3]Mac	yield	viability	yield	viability	yield	viability	yield	viability
1	0.15	0.25	0.34±0.08	81.1	1.04±0.16	73.2	1.28±0.28	69.7	0.25±0.07	55.9
1	0.15	0.5	0.32±0.14	80.6	0.83±0.16	71.9	1.56±0.15	67.5	0.26±0.07	53.1
1	0.25	0.25	1.67±0.34	69.6	3.30±0.25	51.8	3.84±0.63	64.1	3.41±0.79	58.8
1	0.25	0.5	1.94±0.31	54.7	3.34±0.48	58.4	4.77±0.32	55.5	1.70±0.21	36.6
1	0.5	0.25	0.39±0.15	43.4	1.98±0.30	59.8	1.66±0.31	57.7	0.35±0.15	60.6
1	0.5	0.5	1.91±0.62	55.8	1.77±0.40	50.0	1.71±0.29	69.6	1.46±0.24	39.6

[1]Cel: Cellulase Onozuka R-10; [2]Dri: Driselase; [3]Mac: Macerozyme R-10

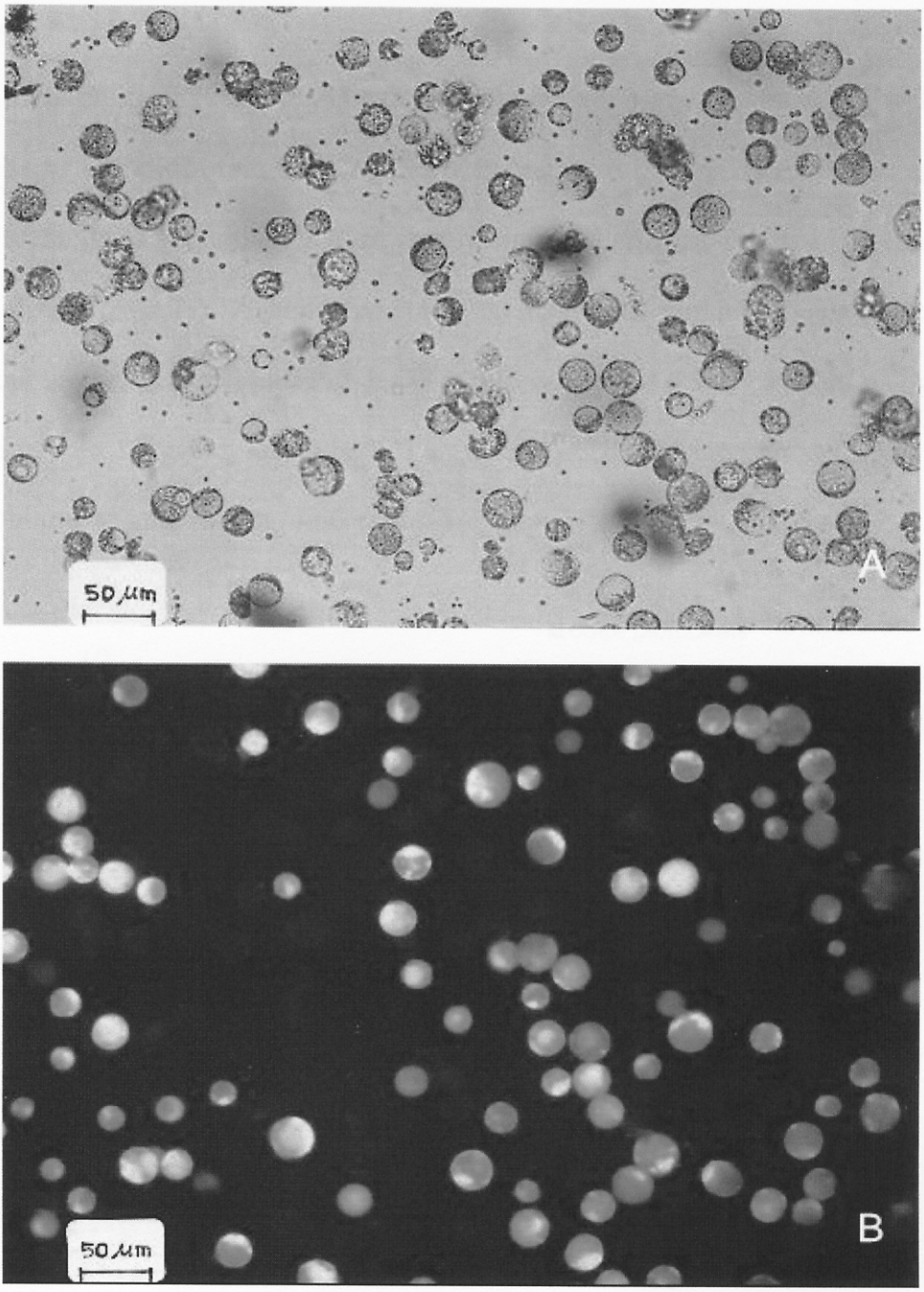


Figure 16 (A) Freshly isolated protoplasts from embryos of oil palm and (B) the same protoplasts when stained with FDA.

4.3.2 Effect of protoplast culture methods and protoplast sources on cell division

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. Methods of protoplast culture significantly affected the relative percentage of cell division ($p \leq 0.05$). A high division percentage was obtained when protoplasts were cultured either in liquid thin layer at a frequency of 6.91±0.54% or using bead technique at a frequency of 5.86±0.33%, whereas cell division obtained from semi-solid culture was the lowest (4.66±0.50%) (Figure 17).

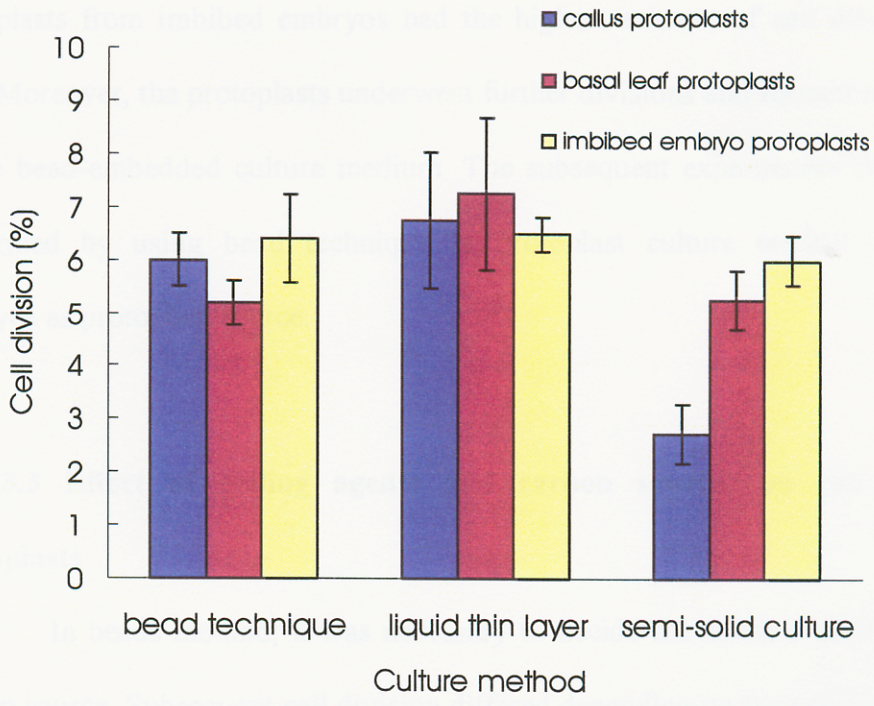


Figure 17 Percentage of cell division of oil palm protoplasts from different sources of explants cultured on MS medium using agarose as gelling agent supplemented with 8.3 μM picloram, 4.5 μM 2,4-D, 8.8 μM BA and 4.6 μM kinetin after cultured for 7 days. Error bars represent ±SE.

Although the highest percentage of cell division was achieved in protoplasts cultured in liquid thin layer technique, continued cell division was not observed in this technique. Cell division in semi-solid culture was lower than that in bead technique. When comparing the 3 different protoplast sources, the type of protoplast sources had no significant influence on subsequent cell division (Figure 17). No difference in % of cell division was detected for imbibed embryo-derived protoplasts in all three culture methods whilst callus-derived protoplasts and basal leaf-derived protoplasts yielded inconsistently. Continued cell divisions were only seen in protoplast cultures derived from the bead embedding and semi-solid procedures. Using these methods, protoplasts from imbibed embryos had the highest amount of cell division (Figure 17). Moreover, the protoplasts underwent further divisions and formed microcolonies in the bead-embedded culture medium. The subsequent experiments therefore were performed by using bead technique as protoplast culture method and imbibed embryos as protoplast source.

4.3.3 Effect of gelling agents and carbon sources on cell division of protoplasts

In beads method, it was necessary to decide the suitable gelling agent and carbon source. Subsequent cell division differed depending on the gelling agents used. The highest division frequency was obtained when imbibed embryo-derived protoplasts were embedded in agarose beads (2.7-4.3%), followed by Gelrite (1.4-2.0%) and sodium alginate (0.7-1.0%) beads (Table 11). Among the 3 different types of carbon source, no significant difference in cell division was observed. However, in agarose bead method, combination of glucose with mannitol or sorbitol gave higher

division frequencies than glucose alone. Since mannitol sometimes gave partial crystallization in medium which might cause damage of colonies, combination of 0.38 M glucose and 0.02 M sorbitol was used as the carbon source for further routine culture of oil palm protoplasts. Activated charcoal had no effect on cell division (data not shown).

Table 11 Percentage of cell division of embryo-derived oil palm protoplasts cultured on MS medium supplemented with 13.6 μM 2, 4-D, 4.4 μM BA and 9.3 μM kinetin using bead technique containing different gelling agents and carbon sources.

Gelling agents	Types of sugars on % cell division (Mean±SE)*		
	Glucose	Glucose+Sorbitol	Glucose+Mannitol
Agarose Type VII	2.7±0.35^{ab}	4.2±0.64^{a}	4.3±0.54^{a}
Gelrite	1.8±0.46^{bc}	2.0±0.31^{bc}	1.4±0.17^{bc}
Na alginate	1.0±0.22^{c}	0.8±0.26^{c}	0.7±0.22^{c}

*Values having superscripts in common are not significantly different at the 0.05 probability level by Tukey test.

4.3.4 Cell division induction

Based on these results, effects of several combinations of plant growth regulators on the initial protoplast culture were tested using the agarose bead method with 0.38 M glucose plus 0.02 M sorbitol as carbon sources. The first division occurred within 24 h of culture and second division was evident after 2-3 days of

culture (Figure 18). The first indication of cell wall regeneration was white fluorescence with Calcofluor White staining around the protoplasts cultured for 24 h after isolation and most protoplasts became oval after 1-2 days. Unequal distribution of fluorescence with Calcofluor White staining of cultured protoplasts was also evident in some protoplasts (figure not shown). The optimum combination of plant growth regulators for initial cell division was found to be 8.3 μM picloram, 4.5 μM 2,4-D, 9.3 μM kinetin and 4.4 μM BA, which resulted in 7% cell division. The other combinations tested were less effective in stimulating cell division (Table 12). It is interesting to note that the concentrations of auxins in the optimum plant growth regulator combination resembled those in callus induction medium.

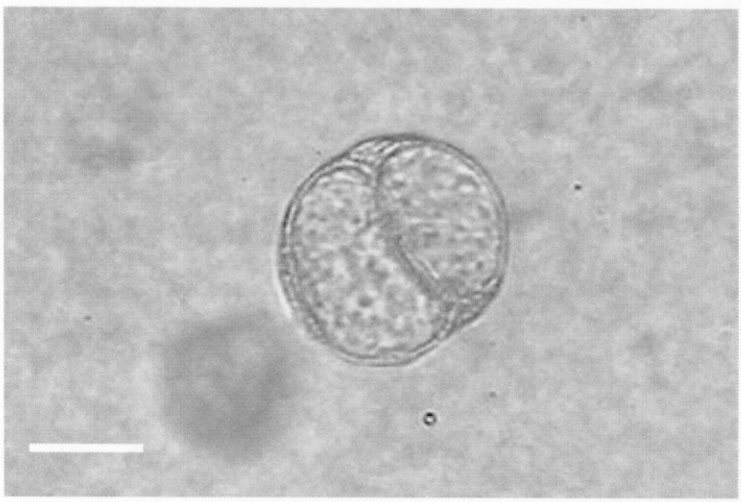


Figure 18 Dividing oil palm protoplasts cultured on MS medium containing 8.3 μM picloram, 4.5 μM 2,4-D, 9.3 μM kinetin and 4.4 μM BA, after 2 days of culture. Bar=10 μm.

Table 12 Percentage of cell division of embryo-derived oil palm protoplasts cultured in agarose bead medium containing various types and concentrations of growth regulators (μM).

Plant growth regulator mixtures (μM)				% Cell division
picloram	2, 4-D	kinetin	BA	(Mean ±SE)*
0	18.1	9.3	4.4	3.7 ± 0.41^{bc}
0	13.6	9.3	4.4	3.5 ± 0.36^{bc}
0	9.0	9.3	4.4	5.3 ± 0.52^{ab}
4.1	9.0	9.3	4.4	4.3 ± 0.49^{bc}
8.3	4.5	9.3	4.4	7.0 ± 0.81^{a}
12.4	0	9.3	4.4	2.8 ± 0.49^{c}

*Values having superscripts in common are not significantly different at the 0.05 probability level by Tukey test.

4.3.5 Cell colony formation

Some of the protoplasts in MS agarose bead medium proliferated into microcolonies (approximately 0.1-0.2 mm in size) after 2-3 weeks of culture (Figure 19). After the first replacement of the liquid medium around the bead with liquid MS medium supplemented with different combinations of plant growth regulators, the media containing dicamba alone or dicamba with 2,4-D showed a more favorable effect on colony formation and gave higher plating efficiencies than those containing

picloram or no auxin (Table 13). However, the plating efficiency in dicamba-containing medium was still low when examined after 4 weeks of culture (0.032%). The gradually reduced osmoticum concentration did not result in microcolony proliferation and they did not grow beyond at the 4th week of culture.

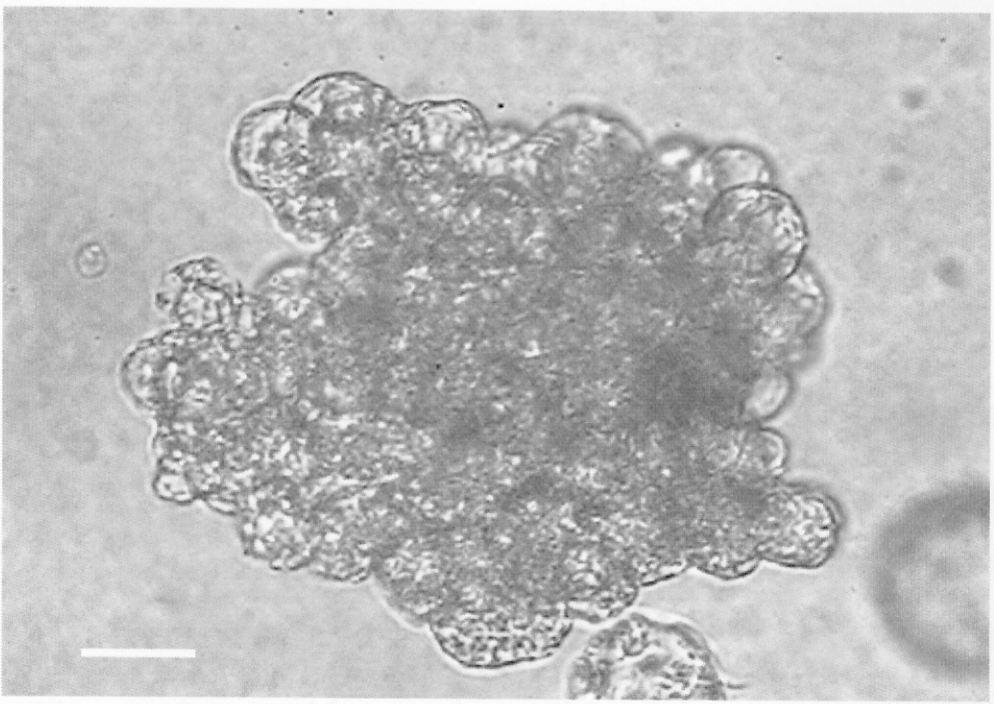


Figure 19 Cell colony formation of oil palm protoplasts cultured in Agarose bead MS medium containing 4.5 μM 2,4-D, 4.5 μM dicamba, 9.3 μM kinetin, 4.4 μM BA and 3.8 μM ABA, after 3 weeks of culture. Bar=20 μm.

Table 13 Plating efficiency of embryo-derived oil palm protoplasts cultured in different mixtures of growth regulators (μM), after 3 weeks of culture.

Plant growth regulator mixtures (μM)							
picloram	2, 4-D	dicamba	kinetin	BA	ABA	0.1% AC[1]	Plating efficiency
0	0	0	9.3	4.4	3.8	-	0.018
0	0	0	9.3	4.4	3.8	+	0.027
0	0	4.5	9.3	4.4	3.8	-	0.032
0	0	4.5	9.3	4.4	3.8	+	0.026
0	4.5	4.5	9.3	4.4	3.8	-	0.030
0	4.5	4.5	9.3	4.4	3.8	+	0.031
4.1	4.5	0	9.3	4.4	3.8	-	0.018
4.1	4.5	0	9.3	4.4	3.8	+	0.015
4.1	0	4.5	9.3	4.4	3.8	-	0.013
4.1	0	4.5	9.3	4.4	3.8	+	0.023

[1]AC: activated charcoal

5. DISCUSSION

5.1 Effect of physical conditions and growth regulators on embryo culture and cell cycle activity

The physiological status of *in vitro* donor material is an important factor for protoplast isolation when cells or tissues are converted to protoplasts. Treatment of embryos prior to seedling induction, callus formation and enzymatic digestion plays an important role in protoplast isolation and culture. As results have shown, an imbibition period, typically 48 h prior to culture of embryos, induces callus and incubation in enzyme improves the efficiency of seedling germination, callus formation and protoplast isolation.

5.1.1 Effect of imbibition on seedling and callus induction

In vivo, imbibition represents the first phase of seed germination, and although its effects on *in vitro* response have been occasionally reported, most studies were performed in relation to germination. Indeed, a controlled imbibition is often used for the initiation of metabolism (Clarke and James, 1991). Rises in water content, cellular volume, and oxygen uptake are involved (Durzan *et al.*, 1971), the latter affecting the ATP level, which is essential for protein and nucleic acid synthesis (Osborne, 1983). Actually, there are many parameters, such as temperature, light and period of imbibition. These parameters interact with each other and affect imbibition on the embryo. For example, in *Eurotia ianata*, the duration of imbibition required to

attain the right degree of hydration was dependent on the temperature (Bai *et al.*, 1999) and in jack pine, light seemed to control germination when the moisture/ water content reached 20% of the seed dry weight, 30 min after the start of imbibition (Durzan *et al.*, 1971). In oil palm, Rabechault *et al.* (1968) 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.

The effect of imbibition is well known to reactivate protein synthesis, transcription, duplication of DNA and mitosis by releasing a blockage of cells in the G1 phase of the cell cycle. Imbibition is therefore the critical pre-treatment for an *in vitro* culture system of oil palm. In this study, imbibition for 48 h is the effective promoter for germinating of seedling and callus formation. However, extended imbibition in water presents two potential problems, namely the possibility of excessive water uptake which could cause damage and subsequent leaching of embryo, or the possibility of water-logging in intercellular air spaces in concomitant with lowering of oxygen supply to the embryo (Nwankwo and Krikorian, 1982). As shown in the results, shorter or longer periods of imbibition than 48 h were less effective for seedling and callus formation. This finding is in contrast with Alang and Fadzillah (1986) who concluded that the oil palm seeds needed an imbibition for 6 days in order to increase seedling germination. However, the pericarp layer was not removed before imbibition in this study.

Removing the pericarp is a necessary step since it influences water uptake by regulating the speed of absorption. Another factor, such as a higher temperature, has been shown to either increase or decrease water uptake depending upon the kind of species (Booth and Bai, 1999). In *Avena fatua*, light, dormancy, and water status

interact with each other to influence germination, and light significantly lowers the germination rates compared to darkness (Hsiao and Simpson, 1971).

The effect of imbibition on the *in vitro* response of zygotic embryos has been considered in a few studies. In *Pinus canariensis*, seeds imbibed for 48 h showed a higher percentage of vigorous embryos than when they were soaked; these vigorous embryos represented 80% of the explants producing buds (Martinez Pulido *et al.*, 1990), while in *P. contorta*, no differences were noted between the imbibed and non-imbibed seeds in the development of adventitious buds (von Arnold and Eriksson, 1981). Nevertheless, the influence of imbibition on the first stage of *in vitro* culture is underestimated, although it has been shown to affect many cellular constituents of the zygotic embryos such as proteins, enzymes, and amino acids. For example, following imbibition, the content of soluble proteins decreased in *P. contorta* (Lammer and Gifford, 1989), lipid proportion and protein accumulation increased in *P. taeda* (Groome *et al.*, 1991), and changes in the activity of enzymes were observed in *Larix laricina* (Pitel and Cheliak, 1986). The response patterns of oil palm embryos to different imbibition treatments may reflect specific requirements and the impact of these components on each developmental pathway. As shown in Appendix 7, lipid proportion and protein accumulation in oil palm embryos increased after imbibition. However, with periods of imbibition longer than 48 h, lipid grains and storage protein in embryos were deformed.

5.1.2 Effect of imbibition on cell cycle activity

These results showed that oil palm embryos removed from mature seeds contain 0.77% G2/M nuclei. In a previous study, the vast majority of nuclei were in

the 2C state, with less than 2% in the S or G2/M phase. Bino *et al.* (1993) hypothesized that during maturation and storage of seeds, the external conditions might affect the 2C/4C ratios of embryonic cells. In quiescent embryos, the protein synthesis, transcription, duplication, and mitosis are stopped and the majority of cells are generally in 2C (Deltour, 1985; Berlyn *et al.*, 1987). In most species, the quiescent embryo is arrested at the pre-synthetic G1 phase which reflects a stringent control over the nuclear division cycle during seed development (Bino *et al.*, 1993; Lanteri *et al.*, 1993). According to Deltour (1985), this arrest in the G1 phase might render seeds more resistant to stress conditions. In some species, however, high amounts of embryonic cells enter the G2 phase during the maturation of a seed (Bino *et al.*, 1993). Amongst the five durations selected in to the present study, various times of embryo imbibition affected relative DNA content, and the zygotic embryos imbibed for 48 h had nuclei in the G2/M phases that varied between 4.5 and 6.8% (Figures 13 and 14a). This treatment gave significantly different effect from all other treatments on the G0/G1 and G2/M phases and from most treatments on the S phases (data not shown). The percentage of G2/M nuclei in non-imbibed embryos was very low compared to that of imbibed embryos. Moreover, the percentage of G2/M phase cells in callus tissues derived from imbibed embryo was higher (2.2–4.8%) than that of non-imbibed embryos.

From a statistical point of view, 48h-imbibed embryos significantly differed from other tissues of *in vitro* culture for the G2/M phase. Amongst the 3 sources of calli selected in this work to study the effects of various combinations of growth regulators and duration of embryo imbibition on relative DNA content, the zygotic embryos imbibed for 48 h of the cell cycle phases were distinguished (Fig. 14). If one

compares the cell cycle phases of embryos in non-imbibed and imbibed embryos, the former had nuclei at the G0/G1 phase in higher number than all other treatments (Figures 13 and 14).

Imbibition is known to initiate synthesis of nucleic acids to repair DNAs, which have been damaged during seed desiccation, maturation, and storage (Osborne, 1983; Bino *et al.*, 1992) and the repair occurs during the first 90 min following imbibition (Osborne, 1983). Furthermore, both developmental and physiological stimuli and the response to environmental stresses may give rise to rapid changes in the amount and organization of nuclear genome. Oil palm has relatively large genome, containing a very high proportion of repetitive DNA (Rival *et al.*, 1997), and in a previous study on three cultivars including *Dura*, *Pisifera* and *Tenera*, the redundancy in repetitive DNA was greater in the dormant *Tenera* cultivar than in that of other cultivars (unpublished data). The apparent pattern in the amount of cell in the G2/M phase of the cell cycle increased when the different physical factors were applied to oil palm embryos.

5.1.3 Effect of growth regulators on callus induction

The growth regulators affecting callus formation in oil palm were auxins. In preliminary experiments, the medium containing 13.6 μM 2,4-D (3 mg/l) provided suitable conditions for callus induction. This auxin concentration is in agreement with previous work reported in bottle palm (Sarasan *et al.*, 2002) and oil palm (Srisawat and Kanchanapoom, 2005). Since the exogenously applied 2,4-D may not be enough to satisfy the auxin requirement of the explants for embryogenic callus formation,

picloram was therefore mixed with 2,4-D as a supporter for inducing embryogenic calli.

The first successful oil palm regeneration via somatic embryogenesis was reported by Rabechault *et al.* (1972). Since then, a few regeneration protocols have been reported both from cell suspension and somatic embryogenesis of oil palm. Among these protocols, the presence of 2,4-D alone or in combination with other growth regulators in the culture medium has been reported to be essential for inducing somatic embryogenesis in oil palm (Kanchanapoom and Domyoas, 1999; Rajesh *et al.*, 2003). However, the effect of 2,4-D on callus induction of different oil palm cultures has been quite variable. In this study, decreasing 2,4-D concentration from 13.6 to 4.5 μM and adding of 0.1% activated charcoal resulted in an increase in frequency of embryogenic callus formation on solid medium, while it did not affect callus induction in liquid medium. As the results have shown, there was an increase in frequency of callus formation in liquid medium as the 2,4-D decreased from 13.6 to 4.5 μM and adding 8.3 μM picloram. Interestingly, two types of callus were induced from oil palm embryos: one was compact and the other was friable. Friable calli induced in liquid medium were larger in size than those on the solid medium, when they were transferred to a basal medium in the absence of, or at low levels of, 2,4-D (4.5 μM) with 0.1% activated charcoal, they developed embryogenic calli (Appendix 8). In contrast, callus induced on solid medium and transferred to a new medium containing low auxins concentration (in particular 2,4-D) and activated charcoal promoted only callus formation.

The synthetic auxin 2,4-D is therefore required in Arecaceae, such as oil palm (Rajesh *et al.*, 2003), bottle palm (Sarasan *et al.*, 2002) and date palm

(Veramendi and Navarro, 1996; Fki *et al.*, 2003) in *in vitro* cultures to promote embryogenic callus formation. Although many studies emphasized the role of 2,4-D in these tissues, more extensive investigation is needed to confirm these findings.

5.2 Flow cytometry in oil palm

When the three species of internal reference plants (*Glycine max* cv. Polanka, *Lycopersicon esculentum* cv. Stupicke and *Zea mays* line CE-777) were used to verify the DNA content of oil palm, the results revealed significant differences in DNA content among the references used. The highest DNA content of oil palm (4.34 pg $2C^{-1}$) was found when using *Zea mays* line CE-777 as an internal reference whereas using *Lycopersicon esculentum* cv. Stupicke and *Glycine max* cv. Polanka as reference plants gave a lower DNA content (3.97 pg $2C^{-1}$ and 4.18 pg $2C^{-1}$, respectively). *Glycine max* cv. Polanka has been described as a reference for Banana (Lysak *et al.*, 1999) but *Lycopersicon* and *Zea* have been little used. In Arecaceae, *Petunia hybrida* (2C=2.85) was mostly used as a reference in flow cytometric analysis such as that of date palm (Siljak-Yakovlev *et al.*, 1996), oil palm (Rival *et al.*, 1997) and coconut palm (Sandoval *et al.*, 2003); however, I could not receive *Petunia hybrida* for this investigation.

Although 2C peak of oil palm did not overlap with those references, the 2C peak of *Zea mays* line CE-777 gave poor reading in peak quality, resulting from interference between the staining solution and the genome of the two species (Amsellem *et al.* 2001), whereas 2C peak of *Glycine max* cv. Polanka was located closer to that of oil palm than *Lycopersicon esculentum* cv. Stupicke, which was

helpful in decreasing the error of DNA content. These results thus showed that *Glycine max* cv. Polanka could be used as an internal reference plant for DNA content analysis of oil palm. Since the technique and equipment used here were different, it might be noted that these results of oil palm DNA content using *Glycine max* cv. Polanka as an internal reference do not resemble those reported by Rival *et al.* (1997). When the references were as an external standard, the results showed that oil palm DNA content using *Glycine max* cv. Polanka as the reference most closely resembled those of Rival *et al.* (1997), which gave a different reading in peak position of oil palm from those obtained with *Lycopersicon esculentum* cv. Stupicke and *Zea mays* line CE-777 an an external standard (Appendix 9).

These results not only coincide with those obtained by Rival *et al.* (1997), but also the DNA contents of cultured tissues were different from that of embryo. DNA contents of calli reported here were significantly higher than that of shoot meristem of seedling whereas Rival *et al.* (1997) reported that DNA contents of cultured tissues were lower than that of plantlets. The occurrence of endopolyploidy in differentiated plant tissues is well known and it is not characterized as an original feature (Kudo and Kimura, 2001) and nuclei distribution depends on types of tissues cultured *in vitro* (Sandoval *et al.*, 2003). The results thus assumed that endopolyploidy might occur in long-term *in vitro* culture of oil palm calli and might have been affected by plant growth regulators used in the culture, particularly 2,4-D. Therefore, long-term *in vitro* culture of calli should not be used as a protoplast source because failure to keep plant regeneration ability and genomic DNA alteration occurs in this culture, whereas DNA content of long-term *in vitro* culture of seedlings and one-month-old calli did not alter.

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 are 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 (Dolezel, 1997). 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 embryos imbibed for 48 h appear to be more efficient material for cell division since the proportion of cells in the G2/M phase was higher than at other imbibition times and could be considered as an initial material. Early germination is dominated by cell elongation, may be advantageous during germination (Price *et al.*, 1998). Mature oil palm seeds with embryos imbibed for 48 h have the highest number of cells in 4C state, hence growth may be accelerated by employing a strategy of increased rate of cell division.

5.3 Protoplast isolation and culture

In this study, imbibed embryos provided larger number of protoplasts with high purity than those from calli and seedlings. This study has 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. They provided

a larger number of protoplasts, higher number of cell division, and cell colony formation than in previous work (Sambanthamurthi *et al.* 1996).

5.3.1 Protoplast isolation

5.3.1.1 Effect of enzymes and times for incubation on protoplast yields

Until now, *Elaeis guineensis* Jacq. has been recalcitrant to regenerate plants from protoplasts. There are only two reports dealing with protoplast production and culture of this economically important plant (Bass and Hughes, 1984; Sambanthamurthi *et al.* 1996). The type and concentration of enzymes and the incubation time are known to greatly influence the quality and quantity of protoplasts. The enzymes combination of 1% Cellulase Onozuka R-10, 0.25% Driselase and 0.5% Macerozyme R-10 was very efficient in releasing protoplasts from the embryonic tissues (4.7×10^6 protoplasts $gram^{-1}$ FW.), whereas for release of protoplasts from other sources, the mixture of enzymes concentration needs to be higher than that for embryos. This is in contrast to the result of Sambanthamurthi *et al.* (1996), who reported that polyembryogenic cultures of oil palm were the most suitable starting materials when they were digested by Pectolyase Y-23 in association with Cellulase and Hemicellulase. The addition of Driselase in the enzyme mixture reported here favored cell wall digestion, in line with data for *Aranda* hybrid mesophyll protoplasts (Loh and Rao, 1985; Koh *et al.*, 1988), and with results by Bass and Hughes (1984), who isolated oil palm protoplasts from cell cultures in a mixture containing 10 g/l Driselase. It should be noted though that the use of Driselase was also found to be harmful for subsequent division of isolated *Pithecellobium dulce* protoplasts (Saxena

and Gill, 1987). The concentration of sorbitol used (109.32g/l) was found to be effective since lysis of protoplasts was not observed.

Suitable incubation time for yield of protoplasts depends on protoplast source. In the present study, incubation for 48 h was found to be effective for protoplast isolation from embryos whereas other sources need to use shorter periods of imbibition.

5.3.1.2 Effect of explants on protoplast yield

It is generally accepted that cell suspension culture is a good source for protoplast culture. However, trials to establish oil palm cell suspension cultures turned out to be difficult and met with limited success (Kanchanapoom and Chourykaew, 1998). Also, embryogenic calli of oil palm were compact and not suitable for protoplast isolation. Therefore, this study tried to isolate protoplasts from other sources.

Of the various explants we tested, embryos imbibed for 48 h were the source tissue of choice since they provided large yield of clean protoplasts. However, the stationary incubation was found to be a critical step when isolating protoplasts from oil palm embryos, whereas isolating protoplasts from other source required agitation. This research has shown that imbibed embryos for 48 h can be used as the source of protoplasts. The development of a protoplast isolation system in oil palm which is recalcitrant to protoplast culture was achieved with this investigation. Also, isolating protoplasts directly from imbibed embryos has several advantages including continuous accessibility to sterile plant material and a relatively limited risk of somaclonal variation (Mills and Hammerschlag, 1994).

5.3.2 Protoplast culture

5.3.2.1 Effect of protoplast sources and culture methods

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 for woody plant species protoplasts (Ortin-Parraga and Burgos, 2003). Protoplast culture of oil palm has been reported from cell cultures (Bass and Hughes, 1984) and polyembryogenic cultures (Sambanthamurthi *et al.*, 1996), but in most cases only microcalli were produced. In the present study, microcalli were able to develop further from imbibed embryo protoplasts after they reached 5-10 cells. It thus should be noted that embryonic cells had large nuclei with a dense cytoplasm and underwent cell division better than non-embryonic cells. Moreover, a more thorough study of the nutritive conditions is needed to overcome the decline in growth of microcalli at an early stage. Research is in progress on this aspect.

The highest cell division was seen in protoplast culture with liquid thin layer procedure. It is generally accepted that a gradual reduction of osmotic pressure supports sustained growth of protoplast-derived cell colonies. Therefore, changing of the liquid medium surrounding the agarose beads in this experiment gave positive results on cell colony formation from protoplasts. Moreover, they developed microcolonies when they were cultured in agarose bead medium whereas other methods of oil palm protoplast cultures were found to be less effective since the rate of further cell division of protoplasts was extremely low (Srisawat and Kanchanapoom, 2005).

5.3.2.2 Effect of gelling agents and carbon sources

One of the important factors for protoplast culture is the kind of gelling agent. As shown in this study, oil palm protoplasts divided better in agarose beads than in sodium alginate or Gelrite beads, indicating that agarose itself may be not as toxic as other gelling agents or that its gelling process is thermo-independent, which improved protection for fragile cells (Karamian and Ebrahimzadeh, 2001). Schnabl and Youngman (1985) hypothesized that the cytoplasmic membranes may be stabilized due to protoplast immobilization, at least during the most critical stage of culture until regeneration of the cell wall begins. Although the mechanism involved in the stimulation of protoplast division is still unclear, immobilization of protoplasts in gelling agents has additional advantages for protoplast culture, such as the improvement of plating efficiency since liquid culture often results in aggregation (Srisawat and Kanchanapoom, 2005) and necrosis of protoplasts.

When comparing the three different sugars as a carbon source, glucose in combination with sorbitol or glucose with mannitol supported a higher percentage of cell division of oil palm embryonic protoplasts than glucose alone. Bass and Hughes (1984) had used a growth medium with 0.3 M glucose for oil palm protoplasts and no divisions were observed. In contrast, Sambanthamurthi *et al.* (1996) employed 400 μM sucrose and 2 μM ribose in addition to growth medium A. However, they reported that glutathione and catalase were important components of the culture medium and protoplast viability was markedly improved in their presence. In the present experiments, the use of sorbitol or mannitol, which is relatively inert metabolically, therefore resulted in stability of cell during culture.

5.3.2.3 Effect of activated charcoal

Activated charcoal had no effects on oil palm protoplast culture. However, advances have been made in understanding the roles of charcoal and the function it fulfils in tissue culture. The discovery that it is certainly operating as an "auxin slow-release agent" is both completely novel and a very considerable advance in understanding the beneficial effects of activated charcoal. This is of extensive application in tissue culture of many plants. From a practical point of view data generated on growth regulator adsorption by charcoal has led to a better mastery of oil palm somatic embryogenesis (Kanchanapoom and Chourykaew, 1998).

5.3.3 Protoplast division and cell colony formation

In the present study, trials to induce cell division of oil palm protoplasts were attempted with different culture media and, after 24 h of culture, embryo-derived protoplasts showed cell division. It is interesting to note that 2,4-D was indispensable for initial cell division whereas picloram was found to be less essential. However, the high initial number of dividing cells (7.0%) was probably due to the supplementation of both picloram and 2,4-D. The concentration of these auxins resembles that of auxin in oil palm callus induction medium (Srisawat and Kanchanapoom, 2005). Picloram, 4-amino-3,5,6-trichloropicolinic acid, is a herbicide with auxin-like properties similar to 2,4-D and it had been successfully utilized for totipotent callus lines in tissue cultures of several grasses and other monocots (Fitch and Moore, 1990), and to induce somatic embryogenesis in pejibaye palm (Valverde *et al.*, 1987) and Canary Island date palm (Lan Huong *et al.*, 1999).

Protoplast cultures of oil palm have been reported in those isolated from cell cultures (Bass and Hughes, 1984) and polyembryogenic cultures (Sambanthamurthi *et al.*, 1996). In most cases, only microcalli were produced. In the present study, cell division was initiated in media containing 2,4-D with picloram or 2,4-D alone, while microcalli were prominently developed in media containing dicamba with 2,4-D or dicamba alone (>30-50 cells). This is an important step since Ochatt and Power (1989) 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.

This research has demonstrated that it is possible to carry out protoplast isolation and culture of oil palm, a recalcitrant plant, which have been difficult to do in the past. The proposed protocol is simple and large amounts of viable protoplasts can be obtained. The next steps should be directed towards identifying the culture conditions leading to optimal plating efficiency and plant regeneration.