2. LITERATURE REVIEW

Taxonomy of oil palm

Class : Angiospermae

Subclass : Monocotyledon

Order : Palmales

Family : Arecaceae (Palmae)

Sub-family : Cocoideae

Genus : *Elaeis*

Species : *Elaeis guineensis*

Scientific name: *Elaeis guineensis* Jacq.

Vernacular names: Oil palm (English); Palm num mun (Thai); Palmier a huile (France); Kelapa sawit (Indonesia)

2.1 Origin and geographic distribution

Oil palm is native to Africa and it is assumed that speciation took place in that continent. However, since all related species classified in the subfamily Cocoideae have a South American origin (except perhaps the coconut, *Cocos nucifera*), the archetypal ancestor may have been indigenous to the Americas. Oil palm occurs in palm groves throughout the tropical rain-forest area of West Africa between 10°N and 10°S of the equator. However, most of these groves show signs of human interference

and probably owe their origin to man. Oil palm has played a major role in the village economy throughout West Africa for many centuries.

Oil palm was introduced to South America with the slave trade. In fact the original description of Jacquin in 1763 was based on a specimen growing in Martinique, semi-wild groves are reported in coastal regions of Brazil around Belem. The oil palm was introduced into South-East Asia in 1848 through the Botanical Garden of Bogor, Indonesia. Second generation and third generation descendants from the originally introduced material were used as planting material for the first oil palm estates in Sumatra (since 1911) and Malaysia (since 1917) and have given rise to the Deli *Dura* breeding population.

2.2 Description of *Elaeis guineensis* Jacq.

A perennial monoecious, erect, one-stemmed palm-tree, normally 20-30 m high. Adventitious root system, forming a dense mat in the upper 35 cm of the soil, with only a few roots penetrating deeper than 1 m. Stem cylindrical, up to 75 cm diameter, covered with petiole bases in young palms, smooth in older trees (10-12 years old). Juvenile leaves are of lanceolate type, entire to gradually becoming pinnate; mature leaves are spirally arranged, paripinnate, up to 7.5 m long; petiole 1-2 m long, spinescent, clasping the stem at base; leaflets linear, 35-65 cm × 2-4 cm up to 376 per leaf (Figure 1). Inflorescences unisexual, axillary, pedunculate, until anthesis enclosed in 2 fusiform or ovate spathes 10-30 cm long, with flowers 3-merous; male ones with numerous cylindrical spikes forming an ovoid body of 15-25 cm long and bearing flowers with 6 stamens, connate at base, with linear anthers; female ones subglobose,

15-35 cm diameter, with numerous lanceolate, spiny bracts, each subtending a cylindrical spikelet with 10-20 spirally arranged female flowers, each with two rudimentary male flowers; stigma sessile, 3-lobed. Fruits ovoid-oblong drupes, 2-5 cm long, tightly packed in large ovoid bunches with 1,000 to 3,000 fruits (Figure 2); drupes with a thin exocarp, an oleiferous mesocarp and a lignified endocarp containing the kernel with embryo and solid endosperm.

Figure 1 Oil palm plantation at Horticultural Research Center, Suratthani Province.

Figure 2 Mature oil palm fruit bunch, *Tenera* cultivar.

2.3 Botanical information

The genus *Elaeis* consists of three species: the African *E. guineensis*; and two species indigenous to South and Central America, (*E. oleifera* and *E. odora*). *E. guineensis* is the major economic species. Fruits of *E. oleifera* have much lower oil content and are used only locally in its natural area of distribution. Little information

is available on *E. odora*. A particular feature of the oil palm with considerable economic consequences is the occurrence of three natural fruit types under monogenic control, which form also the basis for the classification of oil palms (Figure 3 A-C).

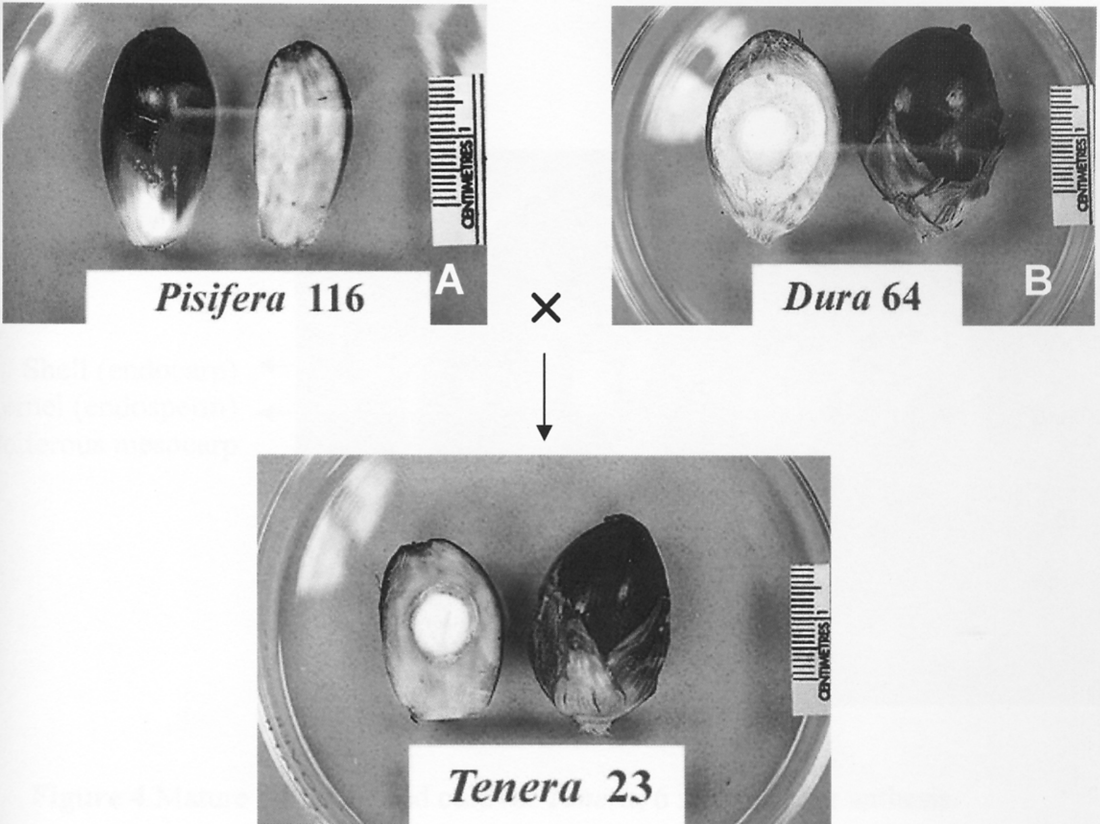

Figure 3 Young oil palm seed cultivars *Pisifera* 116 (A); *Dura* 64 (B) and *Tenera* 23 (C), 3 months after anthesis.

* *Pisifera*: homozygous (sh - sh -) for the absence of an endocarp

* *Dura*: homozygous (sh + sh +) for the presence of a relatively thick endocarp (shell 2-8 mm) and less mesocarp

* *Tenera*: heterozygous (sh + sh -) with a relatively thin endocarp (0.5-4 mm) and more mesocarp

The original Bogor palms and materials derived from them were the thick-shelled types and the population is generally referred to as Deli *Dura*. *Tenera* is preferred as planting material because of more oil bearing mesocarp (60-90% per fruit weight) than *Dura* (20-65% per fruit weight) (Figure 4). Within each fruit type, there is a considerable variation apparently under polygenic control.

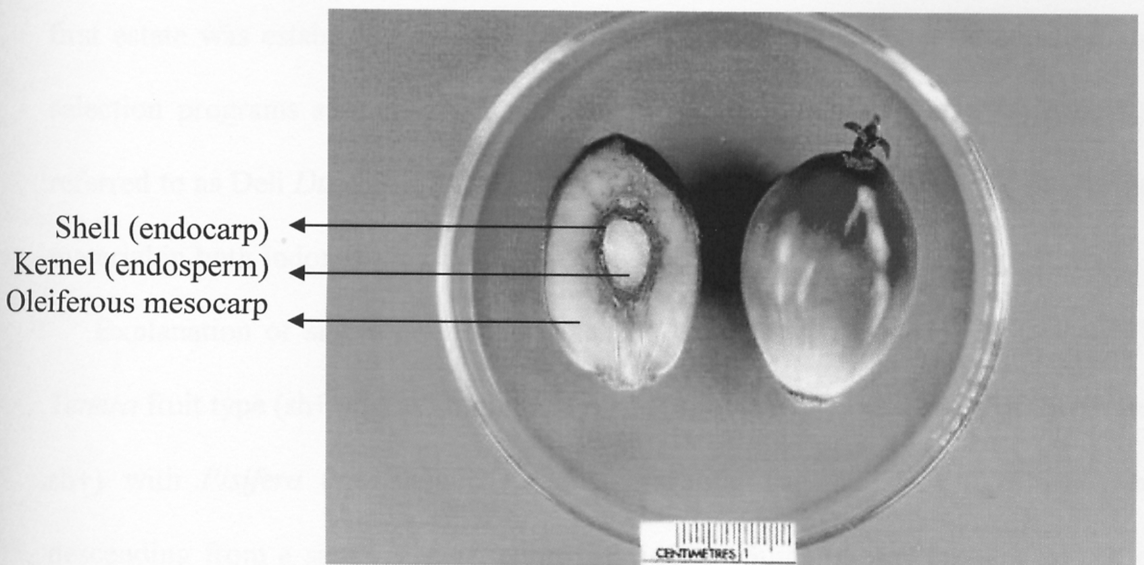

Figure 4 Mature oil palm seed cultivar *Tenera*, 6 months after anthesis.

2.4 Genetic resources

In common with several plantation crops in South-East Asia (notably rubber, cocoa, coffee, tea and cinchona), the genetic origin of the commercial material is rather narrow and to some extent almost accidental. The oil palm industry in Indonesia and Malaysia started with material descended from four palms (thick-

shelled; *Dura*) introduced in the 19th Century in the botanical garden of Bogor (Indonesia). Their simultaneous introduction (probably from Mauritius or Réunion) suggests that the four seeds may well have derived from a single (open-pollinated) fruit bunch. Seeds of these palms and their descendants were widely distributed throughout Indonesia as ornamental palms. Avenue palms in Deli (North Sumatra) supplied the seeds for the first oil palm estates from 1911 onwards. In Malaysia, the first estate was established in 1917. By the early 1920s, a number of breeding and selection programs started, which resulted in improved planting material generally referred to as Deli *Dura*. Until the 1950s, Deli *Dura* was used exclusively as planting material in both Indonesia and Malaysia.

Explanation of single gene inheritance of shell thickness caused interest in the *Tenera* fruit type (sh+ sh-) as commercial material obtained from a cross of *Dura* (sh+ sh+) with *Pisifera* (sh- sh-). Material segregating for the shell thickness gene descending from a single *Tenera* palm (SP 540) in Sumatra was a major source of *Pisifera* for several breeding programs. This palm probably has a common origin with material in the breeding program at Yangambi (Zaïre), descending from nine *Tenera* palms. By the 1960s, major breeding programs in Sumatra and Malaysia concentrated primarily on Deli *Dura* and 'Yangambi' *Pisifera* for production of commercial planting material. Since then, extensive new introductions have been effected from various breeding programs in West Africa (Ivory Coast, Nigeria, Cameroon and Zaïre). In the late 1970s and early 1980s, the Palm Oil Research Institute of Malaysia (Porim) started a systematic program of prospecting and collection from oil groves in West Africa and of *E. oleifera* populations in South and Central America, widening significantly the basis for breeding programs.

In Thailand, in 1968 oil palms were first cultivated at Satoon and Krabi Provinces, south of Thailand, and are still increasing annually throughout the southern region. These oil palm seedlings were derived from Malaysia and revealed the uncorrected phenotype of *Tenera* after cultivation because most of plants produced the fruits with *Dura*-like characters despite some plants displaying those of *Tenera*. Moreover, oil palm agriculturists often cultivated new oil palm plants from the germinated seeds, second filial generation. These problems caused the palm oil industry in Thailand to grow slowly and to be of low value.

2.5 Palm oil

Palm oil is produced from the fruit of the *E. guineensis* Jacq. It is a vegetable oil and therefore does not contain cholesterol. The oil palm fruit yields two types of oil: palm oil from the fleshy mesocarp, and palm kernel oil from the kernel, in the volume ratio 10:1. The two oils differ in composition and properties and, consequently, find rather different applications. Palm oil contains much less saturated fat than palm kernel oil or coconut oil. Palm oil has been used in food preparation for over 5,000 years. Today it is consumed worldwide as cooking oil, margarines and shortening, as an ingredient in fat blends and a vast array of food products. Food manufacturers choose palm oil because it has a distinctive quality, requires little or no hydrogenation, and lengthens the shelf life of products. These advantages are difficult to duplicate at the same cost with polyunsaturated oils, which often have higher market prices and require additional processing for the same characteristics. Ninety percent of all palm oil is used in foods. In South-East Asia, the preferred oil

consumption is clear liquid oil. For domestic use, the liquid fraction palm olein is satisfactory, provided the ambient temperature is above 20°C. Main uses of exported palm oil are margarine, fat used in pastry production and in industrial frying of potato chips, instant noodles and snack foods. Fractions of palm oil are useful in confectionery. Palm stearin, the solid fraction of palm oil, is increasingly used in soap manufacture. Palm-derived fatty acids, mainly commercial grades of stearic and palmitic acids, form an alternative to the traditional products based on tallow.

Palm kernel oil is lauric-type oil similar in composition and properties to coconut oil. Palm kernel oil is also used for industrial purposes, either as an alternative to coconut oil in the manufacture of high quality soaps, or as a source of short-chain and medium-chain fatty acids. These acids are chemical intermediates for the manufacture of fatty alcohols, esters, amines, amides and more sophisticated chemicals, which find a multitude of end-uses, for instance in surface-active agents, plastics, lubricants and cosmetics. The utilization of oil palm by products is currently the subject of research. In recent years, research has shown palm oil can be used to power vehicle, namely bio-diesel.

2.6 Economic and production data

The oil palm is a major oil crop, although taking second place in the world supply of vegetable oil after soybean, and gives the highest output value compared to major oilseed crops, namely rapeseed, soybean and sunflower as shown in Table 1 (Basiron, 2002). The following data are based on FAO (2003). Between 1970 and 2003, world palm oil production increased from less than 2 million tons to over 20 million tons

and still is rising rapidly. South-East Asia is the main area of production, with 76% of the total world palm oil production. By country, production of palm oil in 2003 was for Malaysia 13 million tons, Indonesia 10 million tons, Nigeria 0.9 million tons and Thailand 0.6 million tons.

Table 1 Output values of various oilseed crops.

Crop		Palm oil-Malaysia 2000	Rapeseed-EU 15 1999/2000	Soybean-USA 1999/2000	Sunflower-Argentina 1999/2000
Harvest yield (tons/ha/yr)		18.33	3.18	2.45	1.77
Oil	Oil extraction (%)	18.86	41.70	18.80	37.50
	Oil yield (tons/ha/yr)	3.46	1.33	0.46	0.66
	Price (US$/ton)	310	347	338	392
Non-oil: Meal or kernel	Extraction (%)	5.51	56.80	79.10	39.40
	Oil yield (tons/ha/yr)	1.01	1.81	1.94	0.70
	Price (US$/ton)	63	131	200	106
Output	Total value (US$/ha/yr)	1135.43	696.76	543.27	334.11

Source: (Basiron, 2002)

About half of the world palm oil production (>13 million tons) was accounted for by Malaysia. Besides Malaysia, Thailand also recorded favorable growth in production during this period with registered impressive growth of 9.2% per annum (the 2nd in the world ranking), even though having relatively low hectare, with production increasing from 0.23 million tones in 1990 to 0.56 million tones in 2000 (Table 2) (Basiron, 2002). Nowadays, in 2003, FAO (2003) reported that the palm oil production of Thailand increased to 0.62 million tons and production will continue to increase (Table 3). Among the world's oil palm cultivated area in Table 4, there were about 3.5 million hectares of matured oil palm areas in the year 1990 with an increase to 6.6 million hectares of matured oil palm land in the year 2000.

Table 2 World production of palm oil by major countries (1000t).

Countries	1980	1990	1997	1998	1999	2000	Annual growth rate (%) 1990-2000
Ivory Coast	182	270	240	275	269	266	-0.1
Nigeria	433	580	680	690	720	740	2.5
Colombia	74	226	441	422	475	524	8.8
Ecuador	37	120	203	200	220	250	7.6
Indonesia	691	2413	5380	5006	5900	6950	11.2
Malaysia	2576	6095	9069	8320	10554	10840	5.9
Thailand	13	232	390	355	400	560	**9.2**
PNG	35	145	575	215	270	296	7.4
Others	768	934	866	1197	1173	1371	3.9
Total	4840	11014	17844	16680	19981	21797	7.1

Source: Oil World (various years); MPOB (2000; 2001); FAO (1982); (Basiron, 2002)

Table 3 The 20 highest producing countries of palm oil for the year 2003 (by FAO). *

Selected commodity : Oil of Palm

Selected year : 2003

Rank	Commodity	Production (MT)	Footnote
1	Malaysia	13,180,000	
2	Indonesia	10,100,000	
3	Nigeria	905,000	
4	Thailand	620,000	
5	Colombia	580,000	
6	Papua New Guinea	325,000	
7	Côte d'Ivoire	276,000	
8	Ecuador	243,500	
9	China	217,000	
10	Democratic Republic of the Congo	175,000	
11	Costa Rica	155,790	
12	Cameroon	144,000	
13	Brazil	120,000	
14	Ghana	108,000	
15	Honduras	97,000	
16	Venezuela	70,000	
17	Angola	58,000	
18	Philippines	57,700	
19	Guinea	50,000	
20	Guatemala	44,250	

F = FAO estimate | * = Unofficial figure I Mt = Metric Ton

*(http://www.fao.org/es/ess/top/commodity.jsp?commodity=257&lang=EN&year=2003)

Table 4 World mature areas of oil palm (1000 ha).

Countries	1980	1990	1997	1998	1999	2000	Annual growth rate (%) 1990-2000
Ivory Coast	100	128	159	135	136	139	0.8
Nigeria	220	270	355	358	358	360	2.9
Colombia	27	81	119	121	128	134	5.2
Indonesia	230	617	1517	1647	1840	2014	12.6
Malaysia	805	1746	2455	2597	2857	2941	5.5
Thailand	15	94	146	148	155	199	**7.8**
Others	151	527	602	596	521	731	3.3
Total	1756	3463	5353	5602	5995	6563	6.6

Source: Oil World (various years); MPOB (2000; 2001). (Basiron, 2002)

Although over half of the matured oil palm land was also accounted for by Malaysia (2.94 million hectares; in the year 2000), they had only increased at the rate of 5.5% per annum which is slightly below the world average. On the contrary, Thailand has shown the second ranked increase of 7.8% per annum in matured oil palm area growth rate (Basiron, 2002). Meanwhile, the export value of palm kernel oil of Thailand was ranked third in the world with a value of 19168 US$ in the year 2002 (Table 5). Although the current average yield of palm oil in Thailand is 2.8 tons per hectare and Malaysian palm oil is 3.6 tons per hectare, it is predicted that with the development of new high yielding planting materials including clonal planting materials the industry can achieve higher yields than in the past. The experiments of the research institute and university laboratory may support the procedure for successful production of high yielding oil palm clone.

Table 5 The 20 highest exporter countries of palm kernel oil for the year 2002 (by FAO). **

Selected commodity: Oil of Palm Kernels **Selected year:** 2002

EXPORTS: Countries by commodity (sorted by value)

	Country	Quantity (Mt)		Value (000 US$)		Unit value (US$)
1	Malaysia	670764		277380		414
2	Indonesia	738416		256234		347
3	Thailand	60083		19168		319
4	Papua New Guinea	23243	*	8381	*	361
5	Colombia	17169		6499		379
6	Netherlands	11764		5896		501
7	Singapore	11321		4662		412
8	United States of America	3134		2980		951
9	Benin	4600	*	2800	F	609
10	Germany	3044	*	2603		855
11	Costa Rica	3039		1587		522
12	Denmark	2064	*	1413		685
13	Saudi Arabia	1785		1216		681
14	Guatemala	1994		971		487
15	Democratic Republic of the Congo	1400	F	850	F	607
16	United Kingdom	1097		764		696
17	Honduras	1625		743		457
18	Belgium	1427		708		496
19	Ecuador	1098		457		416
20	Spain	1082		407		376

F = FAO estimate | M = Data not available | T = Trend calculation | * = Unofficial figure | Mt = Metric Ton

** (http://www.fao.org/es/ess/toptrade/trade.asp)

2.7 Imbibition

Imbibition is the process by which the seed rehydrates by absorbing up large volumes of water, swelling to many times its original size. It has been hypothesized that a cause of seed dormancy is due to certain factors limit water uptake by the embryo. In essence the embryo is unable to take up sufficient water during imbibition for germination. This hypothesis is based on the observation that there is a marked difference in the uptake of water by caryopses and embryos of dormant and non-dormant plant seed, imbibition should eliminate the problem.

Seed imbibition is a critical step in the establishment of an *in vitro* culture system, and its importance is often underestimated. *In vivo*, imbibition represents the first phase of seed germination, and although its effects on *in vitro* response have been occasionally raised, most studies were performed in relation to germination. Indeed, a controlled imbibition is often used for the initiation of metabolism (Clarke and James, 1991). A rise in water content, cellular volume, and oxygen uptake is involved (Durzan *et al.*, 1971), the latter affecting the ATP level, which is essential for protein and nucleic acid synthesis (Osborne, 1983). There are many parameters, including temperature, light, and duration, that influence the effect of imbibition on the embryo, and these parameters interact with each other. 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. More than 70% of the weed seed germinated when the relative humidity around the seed was >98.5%, which is vapor in equilibrium with water potentials greater than

approximately -2.0 MPa. Bouaziz and Bruckler (1989) hypothesized that water potentials above -0.9 MPa are not substantially different in influencing imbibition.

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. Another attempt was made in 1986 by Alang and Fadzillah. The most effective imbibition time for germination of oil palm was 6 days. However, 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 the embryo (Nwankwo and Krikorian, 1982).

Imbibition is known to reactivate protein synthesis, transcription, duplication and mitosis by releasing the blockage of cells in G0/G1 (Deltour, 1985; Berlyn *et al.*, 1987). Few data are available concerning the impact of imbibition on absolute DNA amount, although DNA plasticity has been shown to occur in relation to germination and environmental factors (Ceccarelli *et al.*, 1997).

2.8 Flow cytometry

Flow cytometry is a general method for rapidly analyzing large numbers of cells individually using light-scattering, fluorescence and absorbance measurements. The power of this method lies both in the wide range of cellular parameters that can be determined and in the ability to obtain information on how these parameters are distributed in the cell population. Flow cytometric assays have been developed to determine both cellular characteristics such as size, membrane potential, and

intracellular pH, and the levels of cellular components such as DNA, protein, surface receptors, and calcium. Measurements that reveal the distribution of these parameters in cell populations are important for plant biotechnology, because they better describe the population than the average values obtained from traditional techniques (Rieseberg *et al.*, 2001).

2.8.1 Principles of flow cytometry

A flow cytometry system consists of five main operating units: a light source (mercury lamp or laser), flow cell, optical filter units for specific wavelength detection over a broad spectral range, photodiodes or photomultiplier tubes for sensitive detection of the signals of interest, and a data processing and operating unit. A cell suspension is injected into the flow cell where the cells pass one after another across a laser beam (or mercury lamp light) that is orthogonal to the flow. This is achieved by hydrodynamic focusing of the sample stream, wherein the sample stream is injected into the sheath stream inside the flow cell. Using this technology, it is possible to detect up to 10,000 cells/second. In some cytometers, the laser beam is split to give both exact cell size determination via transmission measurement and velocity determination (Eisert, 1981). When it impacts a cell, the excitation light is scattered in both forward and sideways directions. The forward-scattered light provides information on the size of the cells and can be detected without further manipulation. The sideways-scattered light is affected by several parameters, including granularity, cell size and cell morphology. The resulting fluorescent light is processed through the photomultiplier to the data processing system; and the resulting data are analyzed by the cytometer software.

2.8.2 The use of reference plant

Flow cytometry has been applied to estimate nuclear DNA content in several plant species (Bennett and Leitch, 1995; Dolezel *et al.*, 1998). It offers a simple, rapid, accurate and convenient method for determination of ploidy levels of DNA assessments and analysis of the cell cycle of large cell populations (Winkelmann *et al.*, 1998; Dolezel, 1991). However, the choice and correct use of reference standards is a criterion which has been largely neglected (Dolezel *et al.*, 1998). For oil palm, discrepancies are quite large and might be attributable not only to the difference among the techniques, but also to the type of standard used for estimation (Rival *et al.*, 1997). 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 (Sandoval *et al.*, 2003). In some monocotyledons, such as banana, *Glycine max* cv. Polanka was used as internal reference marker (Lysak *et al.*, 1999). The majority of previous works used reference plants as an internal marker. However, when the references were used as an internal marker, Amsellem *et al.* (2001) reported the interference between the staining solution and the genome of the two species which resulted in poor reading in peak quality. It is therefore necessary to clearly define that the reference plants should be used as internal references in each plant.

2.8.3 Cell cycle analysis

Plant morphogenesis involves both the tight control and the co-ordination of proliferative activity through control of the cell cycle in meristematic tissues. The cell cycle can be classified into four steps: the S phase where DNA replication takes place,

which is preceded by the G0/G1 phase and followed by the G2 phase and mitosis (M). The repartition of cells in the different phases of the cell cycle can be a good indicator of cell-division activity for a tissue culture *in vitro* (Winkelmann *et al.*, 1998; Yanpaisan *et al.*, 1999). This was accomplished by measuring nuclear DNA content by means of flow cytometry (Figure 5). In coconut, rapid assessment of ability of tissues cultured *in vitro* to divide has been described as a simple monoparametric flow cytometric method (Sandoval *et al.*, 2003). Therefore this method will make possible a rapid observation of the effect of any given treatment and appears to be a useful tool for a more effective monitoring of the meristematic tissues or protoplasts sources (Yanpaisan *et al.*, 1999).

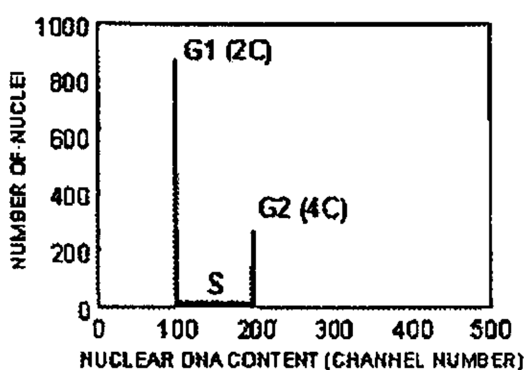

Figure 5 Pattern of cell cycle activity from flow cytometric histogram.

2.8.4 Genomic DNA alterations

At present, clonal propagation of oil palm through tissue culture is common (de Touchet *et al.*, 1991; Teixeira *et al.*, 1995; Aberlenc-Bertossi, 1999; Rajesh *et al.*, 2003) and plant regeneration is mostly achieved through callus or cell suspension cultures. Genome size of these cultured tissues can be altered through changes in the

chromosome number. Oil palm micropropagation through somatic embryogenesis is now used on a large scale. Nevertheless, a proportion of somatic-embryo-derived oil palms has been found to display the "mantled" somaclonal variant phenotype (Jaligot *et al.*, 2000; Matthes *et al.*, 2001), which affects the structure of flowers of both sexes. 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). Although there are many potential markers to investigate genomic DNA alteration of oil palm such as AFLPs (Matthes *et al.*, 2001), difficulties have been encountered when using this procedure on a large scale (Rival *et al.*, 1997).

2.9 Fundamental of plant cell culture

2.9.1 Plasticity and totipotency

Two concepts, plasticity and totipotency, are central to understanding plant cell culture and regeneration.

Plants have developed a greater ability to endure extreme conditions and predation than have animals. Many of the processes involved in plant growth and development adapt to environmental conditions. This plasticity allows plants to alter their metabolism, growth and development to best suit their environment. Particularly important aspects of this adaptation, as far as plant tissue culture and regeneration are concerned, are the abilities to initiate cell division from almost any tissue of the plant and to regenerate lost organs or undergo different developmental pathways in response to particular stimuli. When plant cells and tissues are cultured *in vitro* they

generally exhibit a very high degree of plasticity, which allows one type of tissue or organ to be initiated from another type. In this way, whole plants can be subsequently regenerated. This regeneration of whole organisms depends upon the concept that all plant cells can, given the correct stimuli, express the total genetic potential of the parent plant. This maintenance of genetic potential is called "totipotency". Plant cell culture and regeneration do, in fact, provide the most compelling evidence for totipotency.

In practical terms though, identifying the culture conditions and stimuli required to manifest this totipotency can be extremely difficult and is still a largely empirical process.

2.9.2 The culture environment

When cultured *in vitro*, all the needs, both chemical and physical, of the plant cells have to met by the culture vessel, the growth medium and the external environment (light, temperature etc.). The growth medium has to supply all the essential mineral ions required for growth and development. In many cases (as the biosynthetic capability of cells cultured *in vitro* may not replicate that of the parent plant), it must also supply additional organic supplements such as amino acids and vitamins. Many plant cell cultures, as they are photosynthetic, also require the addition of a fixed carbon source in the form of sugar (most often sucrose). One other vital component that must also be supplied is water, the principal biological solvent. Physical factors, such as temperature, pH, the gaseous environment, light (quality and duration) and osmotic pressure, also have to be maintained within acceptable limits.

2.9.3 Plant cell culture media

Culture media used for the *in vitro* cultivation of plant cells are composed of three basic components:

1) essential elements, or mineral ions, supplied as a complex mixture of salts:
2) an organic supplement supplying vitamins and/or amino acids; and
3) a source of fixed carbon; usually supplied as the sugar sucrose.

For practical purposes, the essential elements are further divided into the following categories:

1. macroelements (or macronutrients);
2. microelements (or microelements); and
3. an iron source.

Complete plant cell culture medium is usually made by combining several different components, as outlined in Appendix 1 and 2.

2.9.4 Carbon sources

Sucrose is cheap, easily available, readily assimilated and relatively stable and is therefore the most commonly used carbon source. Other carbohydrates (such as glucose, maltose, mannitol and sorbitol) can also be used, and in certain circumstances may prove superior to sucrose.

2.9.5 Gelling agents

Media for plant cell culture *in vitro* can be used in either liquid or solid forms, depending on the type of culture being grown. For any culture types that

require the plant cells or tissues to be grown on the surface of the medium, it must be solidified (more correctly termed "gelled"). Agar, produced from seaweed, is the most common type of gelling agent, and is ideal for routine applications. However, because it is a natural product, the agar quality can vary from supplier to supplier and from batch to batch. For more demanding applications, a range of purer gelling agents are available. Purified agar or agarose can be used, as can a variety of gellan gums.

2.9.6 Plant growth regulators

Plant growth regulators are the critical media components in determining the development pathway of plant cells. The plant growth regulators used most commonly are plant hormones or their synthetic analogues. There are five main classes of plant growth regulator used in plant cell culture, namely auxins, cytokinins, gibberellins, abscisic acid and ethylene. Some classes of plant growth regulator will be briefly looked at.

2.9.6.1 Auxins

Auxins promote both cell division and cell growth. The most important naturally occurring auxin is IAA (indole-3-acetic acid), but its use in plant cell culture media is limited because it is unstable to both heat and light. 2,4-Dichlorophenoxyacetic acid (2,4-D) is the most commonly used auxin and is extremely effective in most circumstances. Other auxins are available, and some may be more effective or potent than 2,4-D in some instances.

2.9.6.2 Cytokinins

Cytokinins promote cell division. Naturally occurring cytokinins are a large group of structurally related (they are purine derivatives) compounds. Of the naturally occurring cytokinins, two have some use in plant tissue culture media. These are zeatin and 2iP (2-isopentyl adenine). Their use is widespread as they are expensive (particularly zeatin) and relatively unstable. The synthetic analogues, kinetin and BAP (benzylaminopurine), are therefore used more frequently. Non-purine-based chemicals, such as substituted phenylureas, are also used as cytokinins in plant cell culture media. These substituted phenylureas can also substitute for auxin in some culture systems.

2.9.6.3 Abscisic acid

Although abscisic acid (ABA) inhibits cell division, it is a few used in plant tissue culture to promote distinct developmental pathways such as somatic embryogenesis.

Generalization about plant growth regulators and their use in plant cell culture media have been developed from initial observations made in the 1950s. There is, however, some considerable difficulty in predicting the effects of plant growth regulators: this is because of the great differences in culture response between species, cultivars and even plants of the same cultivar grown under different conditions. However, some principles do hold true and have become the paradigm on which most plant tissue culture regimes are based.

Auxins and cytokinins are the most widely used plant growth regulators in plant tissue culture and are usually used together, the ratio of the auxin to the

cytokinin determining the type of culture established or regenerated. A high auxin to cytokinin ratio generally favours root formation, whereas a high cytokinin to auxin ratio favours shoot formation. An intermediate favours callus production.

2.10 Review of oil palm *in vitro* culture works

The application of tissue culture to oil palm production has been ongoing for three decades since the first successful regeneration of plantlets from leaf tissue was reported by Rabechault *et al.* (1972). Ever since, several investigations have improved upon this method. The technique of producing somatic cell embryos through a callus intermediary has been used (Durand–Gasselin *et al.*, 1990). The use of explants for the regeneration of oil palm using tissue culture procedure has been described and summarized by Esan (1992), whereas the use of embryos for embryoid induction and regeneration of oil palm has been described in many studies (Kanchanapoom and Domyoas, 1999; Rajesh *et al.*, 2003). Te-chato *et al.* (2003) reported the regeneration of oil palm from unopened leaf-derived embryogenic callus. Most research works have reported successful regeneration from somatic embryogenesis. Some related species such as date palm (Veramendi and Navarro, 1996), coconut (Cueto *et al.*, 1997) and bottle palm (Sarasan *et al.*, 2002) have also been reported to undergo regeneration from somatic embryogenesis. Aberlenc-Bertossi *et al.* (1999) reported the regeneration of oil palm from embryogenic suspension culture. Cell suspension culture of some related species has been described such as date palm (Fki *et al.*, 2003). Explants were obtained from shoot apices of unopened young leaves of trees aged 3-25 years or embryos and cultured on Murashige and Skoog (MS) medium

augmented with an auxin usually 2,4-D. After 60 days primary callogenesis occurred, usually from secondary veins. The callus was allowed to multiply for about 6 months to produce secondary callus. The calli produced somatic embryos. The Nigerian Institute for Oil Palm Research (NIFOR), Benin, and the Palm Oil Research Institute of Malaysia (PORIM) have developed many protocols for tissue culture and micropropagation of oil palm (Kwon-Ndung and Misari, 2002). However, little or no effort has been directed towards the use of biotechnological techniques for the production of new cultivars, which can be used to improve the quantity and quality of palm oil.

2.10.1 Protoplast isolation and culture

Protoplasts are plant cells with the cell wall removed. Protoplasts are most commonly isolated from either leaf mesophyll cells or cell suspensions, although other sources can be used to advantage. Two general approaches to removing the cell wall can be taken; mechanical or enzymatic isolation.

Mechanical isolation, although possible, often results in low yields, poor quality and poor performance in culture due to substances released from damaged cells.

Enzymatic isolation is usually carried out in simple salt solution with a high osmoticum, plus cell wall degrading enzymes. It is usual to use a mix of both cellulase and pectinase enzymes, which must be of high quality and purity.

Protoplasts are fragile and easily damaged, and therefore must be cultured carefully. Liquid medium is not agitated and a high osmotic potential is maintained, at least in the initial stages. The liquid medium must be shallow enough

to allow aeration in the absence of agitation. Protoplasts can be plated out to on solid medium and callus produced. Whole plants can be regenerated by organogenesis or somatic embryogenesis from this callus. Protoplasts are ideal targets for transformation by a variety of means.

2.10.2 Early experiments

At the end of the 19th century, protoplasts from higher plants were isolated by mechanical treatment of cells (Klercker, 1892) but owing to the low number of protoplasts obtained by this method, the interest in protoplasts was slight until 1960 when Cocking introduced the enzymatic digestion of the plant cell wall. The enzyme treatment of intact plants and cell suspensions facilitated the preparation of large numbers of free protoplasts, which opened a new research area. In 1970, Nagata and Takebe reported that enzymatically isolated protoplasts from *Nicotiana tabacum* divided and produced callus. The following year Takebe *et al.* (1971) demonstrated that tobacco protoplasts could be cultured in agar plates and induced to form callus from which plantlets could be regenerated.

2.10.3 Importance and use of oil palm protoplast

Protoplasts are naked cells that lack a cell wall. They are spherical with plasmolysed cell content and are contained within a plasmalemma. Protoplasts are excellent material for organelle isolation, as they readily burst in hypotonic solution and release their contents. Furthermore, protoplasts offer several advantages in biochemical studies, e.g. of membranes and cell walls. In principle, each individual protoplast can reform a cell wall, and later initiate either a callus through sustained

divisions, or an embryo, defined as a somatic embryo. This has created opportunities for other biotechnological applications. Cell suspension, for example, opened the way for gene transfer and improved the production, the quality, and therefore the manipulation of cell suspension-derived protoplasts. Recently, protoplast-based biotechnology approaches have been used to complement conventional breeding of many crop species, and plant cultivars could be improved by somatic hybridization and genetic transformation. Oil palm protoplast is therefore an alternative explant to improve elite traits involving somatic hybridization and gene transformation. The use of genetic transformation paves the way for the use of a wide range of beneficial specific genes. Furthermore, this method allows the introduction of only desirable traits to pre-existing and desirable genotypes within a limited exhibition.

2.10.4 Background of genetic transformation

Genetic engineering by transformation offers potential for improvement of the crop. There are many genetic transformation techniques such as *Agrobacterium*–mediated transformation and microinjection. Each method has specific requirements which affect the efficiency. Although *Agrobacterium*–mediated transformation has several advantages such as no requirement for special equipment, techniques or protoplast culture systems, specific gene transfer to target tissues cannot be assured, whereas other successful application of particle-bombardment transformation has been reported for the genetic improvement of some plant species (Mohri *et al.*, 2000). For species that are not susceptible to infection by *Agrobacterium*, micro-injection by delivery of DNA to plant cells is currently the most useful technique. Protoplasts facilitate the direct transformation of plant cells by

DNA microinjection and electroporation. However, many characters of agricultural interest are multigenic or ill-defined, and current transformation methodologies allow the integration of only a few foreign genes.

Important oil palm cultivar is producible to high quality and quantity of oil yield. Very interesting traits of oil production have been identified in this cultivar (Moretzsohn *et al.*, 2000; Rance *et al.*, 2001; Phongdara, personal-communication). Oil palm protoplast culture, therefore, allows the transfer of several useful characters. Even if detailed genetic or molecular knowledge of genes encoding for these desired characters is lacking, protoplast fusion is therefore a complementary tool to increase nuclear and cytoplasmic variability and to confer desirable agronomic traits.

2.10.5 Protoplast culture

Through genetic engineering it has become possible to confer new traits on oil palm plants, using *Agrobacterium*-mediated transfer. Since the oil palm has not been transformed by *Agrobacterium*, protoplasts offer an alternative transformation system. The oil palm being a monocotyledon and a woody plant has been recalcitrant to protoplast isolation and culture. Several reports have described transgenic monocot plants from maize (Rhodes *et al.*, 1989), rice (Toriyama *et al.*, 1988) and barley (Zhang *et al.*, 1995) protoplasts. Despite the economic importance of the oil palm there has been very little information on oil palm protoplasts. Bass and Hughes (1984) reported the regeneration of oil palm protoplasts using nurse culture which comprised oil palm cell suspension as the feeder layer and oil palm protoplasts on a filter paper disc. Sambanthamurthi *et al.* (1996) reported the isolation and regeneration of viable protoplasts from polyembryogenic cultures of oil palm and there have been no further

reports since. In woody plants, reproducible methods of plant regeneration have been reported for protoplasts from coffee (Schopke *et al.*, 1987), rubber (Wilson and Power, 1989; Cazaux and Auzac, 1995; Te-chato *et al.*, 2002) and avocado (Witjaksono *et al.*, 1998), whereas reproducible methods of plant regeneration from monocot plants have been reported for rice (Abdullah *et al.*, 1986; Toriyama *et al.*, 1988), maize (Rhodes *et al.*, 1989), wheat (Li *et al.*, 1992), barley (Wang and Lorz, 1994), banana (Assani *et al.*, 2001) and leek (Buiteveld and Creemers-Molenaar, 1994). These monocot successes have been accomplished using suspension cultures as the protoplast source. Since woody protoplasts are rather difficult to get and handle, therefore, the critical value for breeders of protoplasts still needs to be more adequately demonstrated.

During recent decades, plant biology workers have recognized the potential of protoplasts in many experimental systems, since an efficient enzymatic method for protoplast isolation was first established by Cocking (1960). The first attempts at oil palm protoplast culture were made in 1984 by Bass and Hughes. Cell suspension was used as nurse cell supporting cultured protoplasts. Enzyme solutions were composed of a modified CPW salt solution (Appendix 3), containing 0.7 M mannitol, and a solution of 10g/l Driselase. The second attempt at oil palm protoplast culture was made in 1996 by Sambanthamurthi. Polyembryogenic cell cultures on solid medium were used as protoplast source. Enzyme solutions were composed of a solution containing 800 mOsm (kg $H_2O)^{-1}$ sucrose, and various mixtures of Celluclast, Pectinex 3 XL, Pectolyase Y-23, Macerozyme R-10, Hemicellulase and Driselase. The enzyme solution was adjusted to pH 5.7 and incubated for 5 h in the dark. The tissues that are themselves easily regenerable in tissue culture, namely

polyembryogenic tissue of oil palm, were identified as good donor sources for protoplast isolation and culture. The medium supplemented with glutathione and catalase performed better than MS (Murashige and Skoog, 1962), KM (Kao and Michayluk, 1975) and WPM (Woody plant medium; Russell and McCown, 1986) medium. Protoplast division was not observed and the cells aggregated in the liquid culture medium. Although polyembryogenic cultures were used as a feeder layer, these experiments still were not successful. Further division to form microcalli (300-500 cells) occurred at a very low frequency (<0.01%).