Chapter 18
Oil Palm (*Elaeis guineensis Jacq.*) Somatic Embryogenesis

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18.1 Introduction

The oil palm (*Elaeis guineensis*) originated from West Africa. The species is monoecious, producing separate male and female inflorescences on the same palm, in cycles of varying duration. Oil palm has no distinct types or races, the fruit forms (dura, pisifera and tenera) just being the internal fruit structure, fruit morphology (normal, mantled) and pigments in the exocarp (nigrescens, virescens) (Kushairi et al. 2011). The effectiveness of oil palm breeding from seed remains the basis for long term genetic process. However, there is still considerable unexploited genetic variability within the selected crosses, thus inducing interest in vegetative propagation via tissue culture. This method enables true-to-type reproduction of the best genotypes. Through this, further oil yield improvements can be expected, amongst other advantages (Baudouin and Durand-Gassellin 1991).

Cloning or vegetative propagation is advantageous in the multiplication of elite plants. Many plants are cloned via cuttings, bud grafting, marcotting, etc. However, there are plants that cannot be cloned using these methods. These plants do not produce axillary buds or suckers that can be used for cloning. The oil palm, coconut and some rattan species are examples of such plants. Advances in tissue culture or in vitro methods has led to the cloning of these plants. In the past, monocots have been categorized as recalcitrant to the tissue culture process. Through dedicated research and perseverance, it is now possible to clone monocots such as the date palm (Tisserat et al. 1979), coconut (Thanh-Tuyen and Apurillo 1992), rattan (Aziah 1989) and oil palm (Jones 1974; Rabechault and Martin 1976; Paranjothy

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and Othman 1982). The success in producing oil palm clones has spurred oil palm organizations to set up their own tissue culture laboratories. With an average estimated projection of 20% increase in yield by clones compared to commercial seedlings (Zamzuri 2004), making planting clones a potentially good investment.

In the initial attempts at developing tissue culture protocols for oil palm, extensive studies were carried out using excised zygotic embryos as the starting material (Rabechault et al. 1970; Smith and Thomas 1973; Jones 1974). Subsequently, leaf tissues from polybag plants and mature palms were utilized. The tissue culture protocol for cloning oil palm was established in the 1970s (Jones 1974; Rabechault and Martin 1976; Paranjothy and Othman 1982). Since then, most laboratories in the oil palm industry today, have established their own tissue culture media and protocols (Rajanaidu et al. 1997). Owing to the commercial interests of the laboratories, unfortunately details of the culture media has never been revealed despite the availability of the information through published articles and personal contacts. When the floral abnormality, also known as mantling, was first highlighted in 1986, this triggered all the laboratories to review their media and culture protocols to ensure abnormality free cloning. This led to a reduction in the hormone levels used or a total avoidance of them at certain culture stages (Duval et al. 1988), or the replacement of the chlorinated auxin, 2,4-dichlorophenoxyacetic acid (2,4-D) with α-naphthaleneacetic acid (NAA) (Sogeke 1998). Corley et al. (1986) reported that prolonged maintenance of polyembryoid cultures in vitro showed a higher frequency and degree of abnormality, hence many laboratories set a duration limit for maintaining cultures in vitro either by reducing the number of subculture cycles or the number of plantlets produced.

18.2 Explant Preparation

18.2.1 Field Activities

18.2.1.1 Ortet Sampling

An ortet is a source palm from which leaf, inflorescence and root explants are sampled. In this chapter, only the sampling of leaf explants, which is routinely performed, is described. MPOB selects elite ortets from breeding trials based on the palm performance over a four-year period. The main criteria for selecting an ortet are the yield and other genetic traits, such as disease resistance, palm compactness, oil quality, etc. For yield, the minimum standards required for the selection are 50 kg/palm/year oil yield and 27% oil to bunch ratio, recorded over four consecutive years and five analyses, respectively. This is in accordance with the MS 2099:2008, a Malaysian Standard (MS) specification established for oil palm ortet selection for cloning.

Selected ortets are first marked in the field. During sampling, several steps are taken to ensure workers' safety and that the ortets and samples are maintained in good condition. The young leaves or spears are contained within a cylinder of older leaf petioles or leaf cabbage at the centre of the palm canopy. Climbers have to remove thorns at the petiole bases followed by hewing a small wedge underneath each petiole to ease flattening of the frond. To avoid excessive injury to the palm, only several fronds, of about half whorl of the palm canopy, are flattened as a working platform. The older fronds and fruit bunches are removed, leaving only the cabbage or cylinder of younger frond petioles. The exposed fronds above the cabbage are trimmed off and the youngest leaf with fully opened leaflets, conventionally numbered as 1 (Corley and Gray 1976), is identified and labelled using marker pen. Cutting of the cabbage is a critical operation. The oil palm shoot apex is hidden inside the base of the leaf cabbage at the region where the cylinder bulges. The cabbage is cut at ≥ 10 cm above the bulge ends to ensure that the shoot apex within remains intact. The apex is usually located 6–7 cm below this point. The cabbage is cut towards frond number 1. The trimmed cabbage is about 40–70 cm long depending on the ortet age and the number of times the leaf cabbage had been sampled. The cabbage is placed in a bag and tied with a rope before it is lowered to the ground. The cabbage stump left after sampling needs to be protected from pest attack and fungal infection. If the stump surface is flat, a small sunken cut is made as a precautionary step to discharge any accumulated water or moisture during rainy days before covering the stump with a net pegged by several nails (Fig. 18.1).

Cabbage Handling

Proper handling and prompt transportation of the cabbage from field to the laboratory is very important to maintain its freshness. Both cut ends are wrapped with cling film to prevent dehydration. High humidity, high temperature, improper wrapping and dirty condition of the container or vehicle can spoil the cabbage.

18.2.2 Tissue Culture Laboratory Activities

18.2.2.1 Cabbage Processing

Once the cabbage reaches the laboratory, the wrapping material is removed. The cabbage is inspected for freshness, discoloration, breakage or odor. A sour odor usually indicates the cabbage is contaminated. A cabbage in good condition is whitish in color. Before bringing the leaf cabbage into the laminar airflow cabinet (LAC), some cleaning is required. Outer petioles are removed and only petiole of either frond number 1 or 0 (marked earlier at the sampling site) is maintained. Both ends of the cabbage are sawn or trimmed to expose fresh and clean surfaces. All surfaces (petioles and exposed ends) are surface sterilized by swabbing with absolute alcohol before placing the cabbage in the LAC. Since internal leaf spears

Fig. 18.1 Leaf explant sampling. **a** Removing thorns from leaf petioles. **b** Preparing a working platform. **c** Cabbage before cutting. **d** Cabbage stump after cutting. **e** Lowering of cabbage sample to the ground (arrow indicates cabbage). **f** Covering the exposed cut stump with netting

are considered naturally sterile, the leaf explants can be cultured directly on to the media. The cabbage then undergoes frond marking as each leaf explant is tracked throughout the tissue culture process. Basically, frond marking involves numbering the frond in a descending order, from frond number 1, 0, −1, −2, −3 and so forth using labeled pinheads or thumb-tacks (Fig. 18.2a). A longitudinal cut is made to remove the outermost petiole followed by the subsequent petiole until all the internal fronds or leaf spears comprising stacks of young leaflets are exposed (Fig. 18.2b). The uppermost exposed leaf spear is excised first followed by the next exposed spear. The leaf spears are cut into three or four segments of 9 ± 1 cm in length. Each segment is referred to as a zone. Zone 1 is located nearest to the base of the spear. Since the lower zones generate lower callusing rates than the upper zones (Rohani 2001), zone 1 is usually not cultured. This portion is often deposited with the MPOB Molecular Laboratory for DNA banking. The leaf explants at about

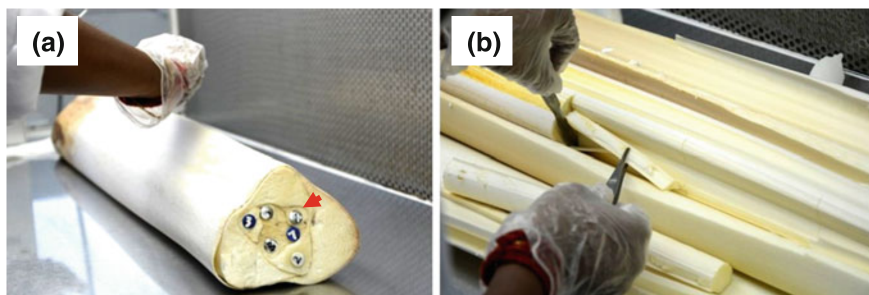


Fig. 18.2 Leaf cabbage. **a** The basal end of the cabbage is marked with labelled pinheads (as arrowhead) (frond −2 to −7). **b** The cabbage is cut longitudinally to expose the leaf spears that are cut into three segments

5 cm from the distal end, is also not cultured to avoid contamination. The leaf spear segments (according to zone and frond number) are placed separately in sterile petri dishes, sealed with cling film and then distributed to the operators for explant cutting and inoculation. At MPOB, fronds −4 to −9 are often used.

18.3 Culture Medium and Condition

18.3.1 Solid Culture

The nutrient formulations were based on modified Murashige and Skoog (1962) as shown in Table 18.1. Nutrient and agar solutions are prepared separately. For both portions, volumes are halved while concentrations of the constituents are maintained. The pH of the nutrient solutions is adjusted to 5.7 while the pH of agar is adjusted to 7.0 before mixing. All media are sterilized by autoclaving at 121 °C under 1.05 kg cm^{-2} pressure for 15 min. The explant and callus cultures are cultured in petri dishes containing 30 ml of callus and embryoid initiation media respectively and incubated under dark condition. Polyembryoid (PE) and shoot development cultures are cultured in 250 ml conical flasks containing 85 ml each of PE proliferation and shoot development medium incubated under 12/12 h light and dark photoperiod. Cultures are maintained at 28 ± 1°C with ± 60% relative humidity.

Callus Induction and Proliferation Media

The nutrient medium composed of components listed in sections A–D, and G–I (Table 18.1) and supplemented with;

(a) 8–10 mg $litre^{-1}$ NAA; or
(b) 5–10 mg $litre^{-1}$ NAA + 0.1–1.0 mg 2,4-D

Table 18.1 Modified Murashige And Skoog (MS) media

Constituents	Concentrations (mg l^{-1})
A. Macronutrients	Modified Murashige and Skoog (1962)
NH_4NO_3	1650
KNO_3	1900
$CaC1_2.2H_2O$	440
$MgSO_4.7H_2O$	370
KH_2PO_2	170
B. Micronutrients	
H_3BO_3	6.2
$MnSO_4.4H_2O$	22.3
KI	0.83
$ZnSO_4.7H_2O$	8.6
$CuSO_4.5H_2O$	0.025
$CoC1_2.6H_2O$	0.025
$Na_2MoO_4.2H_2O$	0.25
NaFeEDTA	37.5
C. Vitamins	
Thiamine.HCl	0.1
Pyridoxine.HCl	0.5
Nicotinic acid	0.5
D. Myo-inositol	100
E. Amino acids (Eeuwens and Blake 1977)	
L-Arginine	100
L-Asparagine	100
F. L- Glutamine	100
G. Glycine	2
H. Sucrose (local refined table sugar)	30,000
I. Agar	7000
J. Gelrite	2500

Embryoid induction and polyembryoid multiplication medium

Calli is placed in embryoid induction medium consisting of the basal nutrients of sections A–D, G–I (Table 18.1) with half amounts of hormone used. This is followed by a gradual decrease in hormone usage in subsequent PE subcultures.

Shoot development (SD) and root initiation (RI) medium

The medium constitutes components from sections A–D, G, H and J (Table 18.1), supplemented with 0.02 mg $litre^{-1}$ NAA.

Root elongation (RE) medium

The medium constitutes components from sections A–D, G and H (Table 18.1) and 0.2 mg litre^{-1} NAA. A volume of 10–12 ml of the RE medium is dispensed into each test tube.

18.3.2 Liquid Culture

18.3.2.1 Liquid Media for Culture Maintenance

The liquid medium consists of components from sections A–C, E and G (Table 18.1) supplemented with 1 mg litre^{-1} 2,4-D and 0.1 mg litre^{-1} NAA. A volume of 20 ml media is dispensed into each 100 ml flask.

18.3.2.2 Liquid Media for Culture Maturation

The liquid medium for maturation contains nutrients listed in sections A–E and G (Table 18.1), a basal medium without plant hormones. A volume of 20 ml media is dispensed into each 100 ml flask.

18.4 In Vitro Culture Protocol

18.4.1 Explant Cutting

To prepare the explant strips, sub-stacks of six leaflets are peeled from the 9 ± 1 cm segment stack and transferred to another sterile petri dish for explant cutting. These leaflets are cut into strips of about 2 mm in width (Fig. 18.3a). Each strip (six-layer stack) is inoculated with one side of the cut edges having direct contact with the callus induction medium (Fig. 18.3b). A 9 cm diameter petri dish can accommodate 18 explants. This process is repeated until all the explants are inoculated. The petri dishes are sealed with cling film and labelled with information such as ortet identification, operator identity, frond number, zone number, medium type and inoculation date. The petri dishes are piled up in a petri dish holder before placing them on racks under continuous darkness for three months. A three-piece petri dish holder can accommodate 42 petri dishes, while a rack-shelf (151.5 × 75.5 cm) can accommodate 24 petri dish holders. Thus, a rack with six shelves can hold 6048 petri dishes (42 × 24 × 6) in total. A cabbage can produce up to ca. 3000 strips (six-layer stacks) in ca. 1000 petri dishes. Therefore, a six-shelf rack can generally accommodate ca. six ortet cabbages.

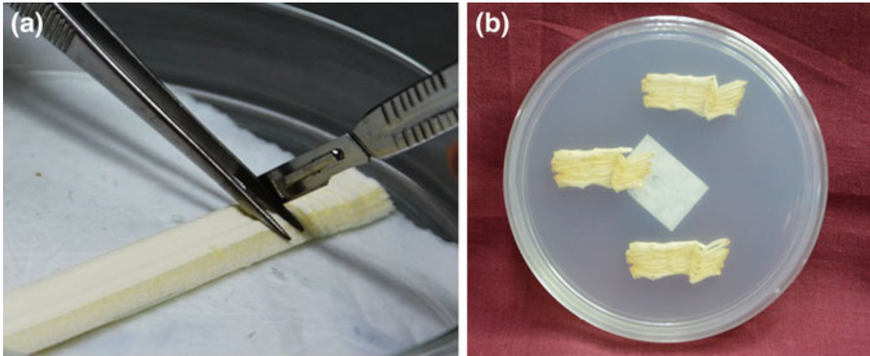


Fig. 18.3 Leaf explants. **a** Six layers of leaf segments are cut into 2 mm explant slices. **b** Three bands of explants comprising of six layers each were placed in a petri dish

18.4.2 Callus Culture

After three months of incubation, the explants are examined for callus formation. Leaf strips with calli are transferred to petri dishes containing fresh embryoid induction medium, while non-callusing explants are subcultured onto fresh callus induction medium at three-monthly intervals for one year before discarding. The callogenic cultures (Fig. 18.4a) are incubated in continuous darkness and subcultured at three monthly intervals for bulking and primary embryoid initiation (Fig. 18.4b). The callogenic cultures are also maintained for one year before discarding.

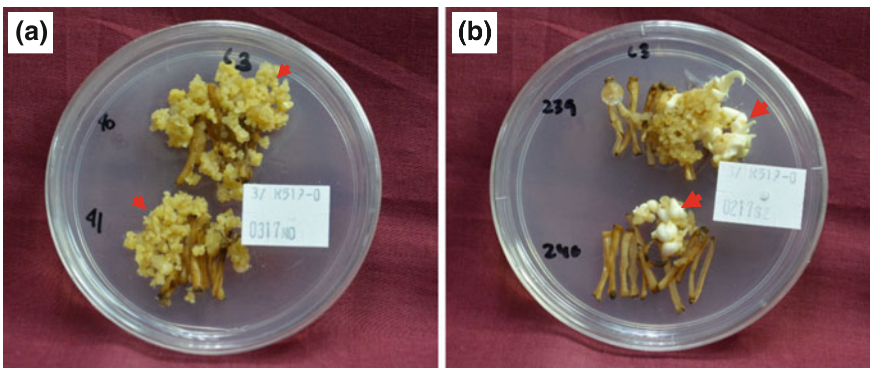


Fig. 18.4 Proliferation of callus. **a** Highly proliferating callus (as arrowheads). **b** Primary embryoids (as arrowheads)

18.4.3 Liquid Culture

Establishment of liquid cultures start with an initial 20 flasks with approximately 0.5 g of cultures each. The duration for establishment of liquid cultures may vary between clones and usually requires more than three subcultures. Weight increment for established clones was about 2 to 4-fold after four subcultures in maintenance media and this varied between clones. After about two to four weeks in maturation media, a weight increment of 2 to 6-fold has been observed. Although MPOB's liquid culture protocol is routinely used, certain genotypes required some modifications of the process. Generally, selection of the suitable callus morphotype, hormone concentration and duration in culture were found to be crucial for the development of liquid cultures at all stages.

The liquid culture method can be divided into three main steps: initiation of liquid culture, maintenance and maturation of liquid culture leading to embryoid regeneration. After these steps, the cultures are transferred back into the solid culture process.

Initiation of Liquid Suspension Culture

All culturing work is carried out in the LAC. For initiation of liquid cultures, friable calli (Fig. 18.5a) and media supplemented with plant hormones are required. Flasks containing the media are weighed prior to subculturing of selected friable calli into the liquid media. The opening of the flask is then briefly flamed before covering with a square plastic sheet and sealed with cling film. The flasks are then usually labelled with Experiment Number, Date of Culture, Weight of cell aggregates (g) and culture number. Similar information is recorded on the record-sheet. The flasks of cultures are incubated on a rotary shaker (Fig. 18.5b) in the dark at 27–30 °C.

Maintenance and Maturation of Liquid Culture

Before subculturing, pre-sterilized sieves with pore sizes of 300 μm and 1 mm, sterile 250 ml jars, liquid media for culture maintenance, liquid media for culture maturation and solid media for formation of embryoids are prepared. All these

Fig. 18.5 Liquid culture. **a** Friable callus used for initiation of liquid cultures. **b** Rotary shakers in dark room for liquid cultures. **c** Smaller aggregates for maintenance of liquid cultures. **d** Larger aggregates (>300 μm) for maturation

apparatus are surface sterilized before placing them into the laminar airflow cabinet. The cultures in flask are poured onto the 300 μm sieve for maintenance process of liquid cultures (Fig. 18.5c) while 1 mm sieve is used for selection of cell aggregates for maturation stage (Fig. 18.5d). Suitable cell aggregates are filtered from cultures trapped by the sieve, then carefully transferred onto fresh respective media. Selected cell aggregates collected from maturation cultures are carefully transferred onto solid media for regeneration. The liquid cultures are incubated in the dark room on a rotary shaker while those that have been transferred onto solid media are incubated in the light room. Both the rooms are maintained at 27–30 °C.

Regeneration of Embryoids

First phase

Prior to conducting this procedure, the LAC is UV-sterilized for 15 min and all the apparatus are surface sterilized. Petri dishes containing solid media, precut sterile filter papers that fit the petri dishes, 1 mm sieves, sterile jars and flasks containing cell aggregates in maturation media are prepared and placed in a trolley next to the LAC. Firstly the petri dishes containing media are single-layered with precut filter papers using forceps. The cell aggregates in the flasks are poured onto the sieves and the flow-through is captured in the jars. The cell aggregates collected on the sieves are weighed and separated into batches of approximately 0.5 g and transferred into the pre-prepared petri dishes using a spatula, while the flow through is discarded. The petri dishes are then sealed with parafilm and appropriately labelled with experiment number, date, weight and the flask number of its origin. The plates are then transferred into the dark room with the temperature set between 27 and 30 °C.

Second phase (approx. after 1 month)

After one month, the plates from the dark room are prepared for harvesting. Only embryogenic calli/primary embryoids are selected and transferred into flasks containing fresh media. The flasks are then sealed and appropriately labelled. The remaining non-embyogenic calli in the plates are returned back to the dark room for another month for further growth. The same procedure is repeated the next month. After the second harvest, all plates are discarded. Primary embryoids obtained from both harvests are further maintained and multiplied on hormone-free media for another 14-18 months. Subculture is performed every two months. Shoots obtained during the multiplication process are transferred to shoot development media. At this stage, the protocol is similar to the solid culture system.

18.4.4 Polyembryogenic Culture

Primary embryoids (Fig. 18.6) that are white to pale yellow in colour with smooth surfaces are detached from the callus mass before being transferred into conical

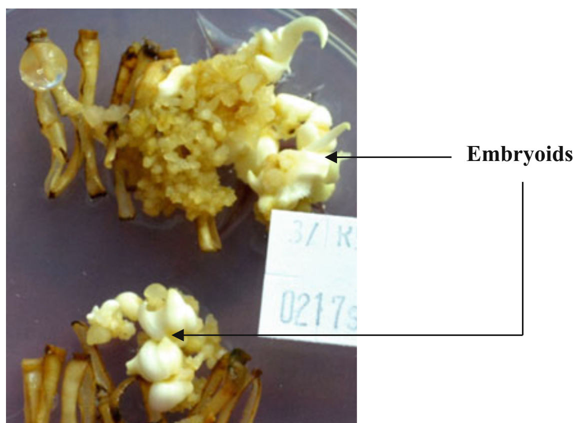


Fig. 18.6 Whitish embryoids with smooth surfaces

flasks containing PE multiplication medium (Table 18.1). These are incubated under a 12/12 h light and dark photoperiod. The flasks are plugged with non-absorbent cotton bungs. The embryoids multiply slowly into bigger compact tissue masses known as polyembryoids (PE) (Fig. 18.7a). Subculture of the PE is carried out every two months. Generally, after the fourth or fifth subculture, multiplication of PE occurs concurrently with shoot formation. The PE masses are cleaned off agar residue, dead or soggy tissues and then cut into smaller clumps of 1.5–2 cm in diameter. Each clump is transferred into a flask for further proliferation, while shoots from PE masses are collected for the next SD stage.

For shoot production, PE cultures are maintained till the maximum 15 subcultures. Upon reaching subculture 11 and thereafter, the PE mass is cut into 0.5 cm clumps and transferred to fresh medium at five clumps per flask. These cultures will be maintained for more than two months to increase shoot formation.

18.5 Shoot Regeneration and Maintenance

18.5.1 Shoot Production and Selection

Quality and quantity are important to attain a substantial number of shoots. Rohani et al. (2000) described that nodular embryoids are more prolific than other embryoid phenotypes. Based on previous records, clones with low number of shoots have either only one or very few embryoid lines. Embryoid phenotypes such as globular or torpedo-shaped did not proliferate readily. As described by Wong et al. (1996), some PE cultures only produce shoots without increasing their embryogenic mass. In some clones, PE cultures did not proliferate up to the maximum 15th subculture

(Table 18.2). Other factors contributing to the low PE culture quantity include contamination, sogginess and poor development of embryoids. At times, shoots derived from the same palm but originating from different explant type (L210 and M210) can lead to significantly different shoot production (Table 18.2).

18.5.2 Shoot Development

Shoots germinated from PE clumps that have attained a height of 2 cm and above are isolated and each shoot base is cut into a V-shape tip before transferring to SD medium. The V-cut was to avoid wounding if the shoot apex which is located at the center of the base and to provide a fresh contact surface to the medium. Usually, ten shoots are transferred into a 250 ml conical flask containing 85 ml SD medium. Each flask is covered with a non-absorbent cotton bung. The flasks are placed directly on a rack in the light room. After 2–3 months, most of the shoots would have attained a height of 6–7 cm with emerging roots (Fig. 18.7b) and are ready for the root elongation stage. Shoots without roots will be subcultured into fresh SD medium.

18.6 Root Induction and Elongation

18.6.1 Root Development in Test Tube

Shoots with roots or emerging roots are transferred into test tubes containing 10 ml liquid RE medium each (Fig. 18.7c).

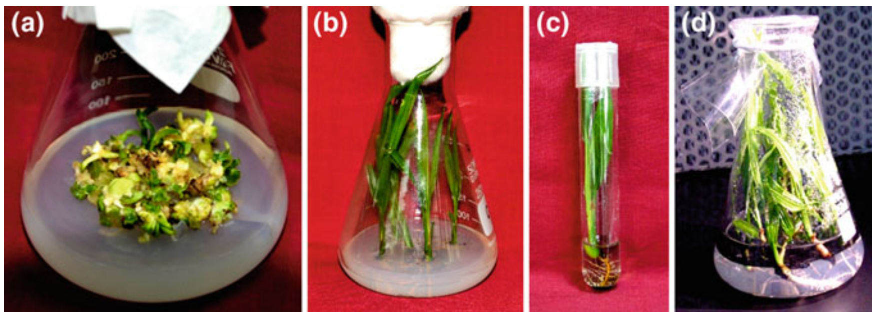


Fig. 18.7 **a** Polyembryoids **b** Shoot Development **c** Root development in test tube. **d** Root development using Double Layer technique

Table 18.2 Clonal shoot production

Clone	Explant type	Ortet ID*	Last subculture number	No. of shoots produced
L207	Leaf	0.827/133	8	45
L208	Leaf	0.827/115	15	393
L210	Leaf	0.189/141	13	160
L233	Leaf	0.827/107	15	1897
L234	Leaf	0.827/65	15	1590
L235	Leaf	0.827/25	13	2750
L236	Leaf	0.187/12	15	29,115
L238	Leaf	0.827/221	13	282
L240	Leaf	0.827/62	13	420
L241	Leaf	0.179/128	15	374
L242	Leaf	0.179/3	15	860
L245	Leaf	0.189/396	8	120
L246	Leaf	0.189/3443	11	40
M210	Male inflorescence	0.189/141	15	17,519
M224	Male inflorescence	0.184/466	14	164
F223	Female inflorescence	0.189/289	6	19

Note *Elite *tenera* ortets

18.6.2 Root Development via Double Layer (DL) Technique

This alternative method is simple and quick. About 50 ml of liquid RE medium, in a dispensing flask is aseptically poured into each flask containing intact shoots, overlaying the solid SD medium (Fig. 18.7d). The cultures are incubated in the light. Two to three months after the addition of the liquid medium, most ramets would have generally exhibited shoot and root elongation. The growth varies among plantlets due to competition for nutrients, space and light. Poor growing plantlets are either culled or subcultured into fresh medium in test tubes for further elongation. This DL method is recommended as a means to mitigate labour shortage when processing high volume of cultures at the shoot development stage. Zamzuri (1998, 2000) showed that by using the DL rooting method, work output could be increased by 18-fold.

Fig. 18.8 **a** Cleaning and **b** removing wilted leaves of hardened shoots

18.6.3 In Vitro Acclimatization

Ramets with a height of more than 12 cm (at the two- to three-leaf stage) and good rooting system are ready for in vitro hardening in the nursery. Rooted cultures still under sterile condition in culture vessels are transferred to pre-nursery with 80% shade for acclimatization for four days. Their vessel caps are opened on the third day and transplanting commences on the fifth day.

18.7 Nursery

18.7.1 Transplanting Stage

The hardened shoots are removed from their test tubes, washed (Fig. 18.8a and b) and soaked in water before transplanting into compacted compost clump covered with netted cloth known as Jiffy-7 pots that are pre-soaked in water. Transplanted ramets are labeled, arranged neatly in perforated trays and placed on a raised platform in the pre-nursery with ca. 75% shade. General maintenance involve irrigation, weekly application of foliar fertilizer, weeding, pest control, culling etc. After four to five months, the ramets are sent to the main nursery and transferred into larger polybags under temporary shading conditions before exposing them to direct sunlight. General nursery maintenance is applied as recommended for DxP seedlings (Kushairi and Rajanaidu 2000). The whole protocol for solid and liquid culture systems is summarized in Fig. 18.9.

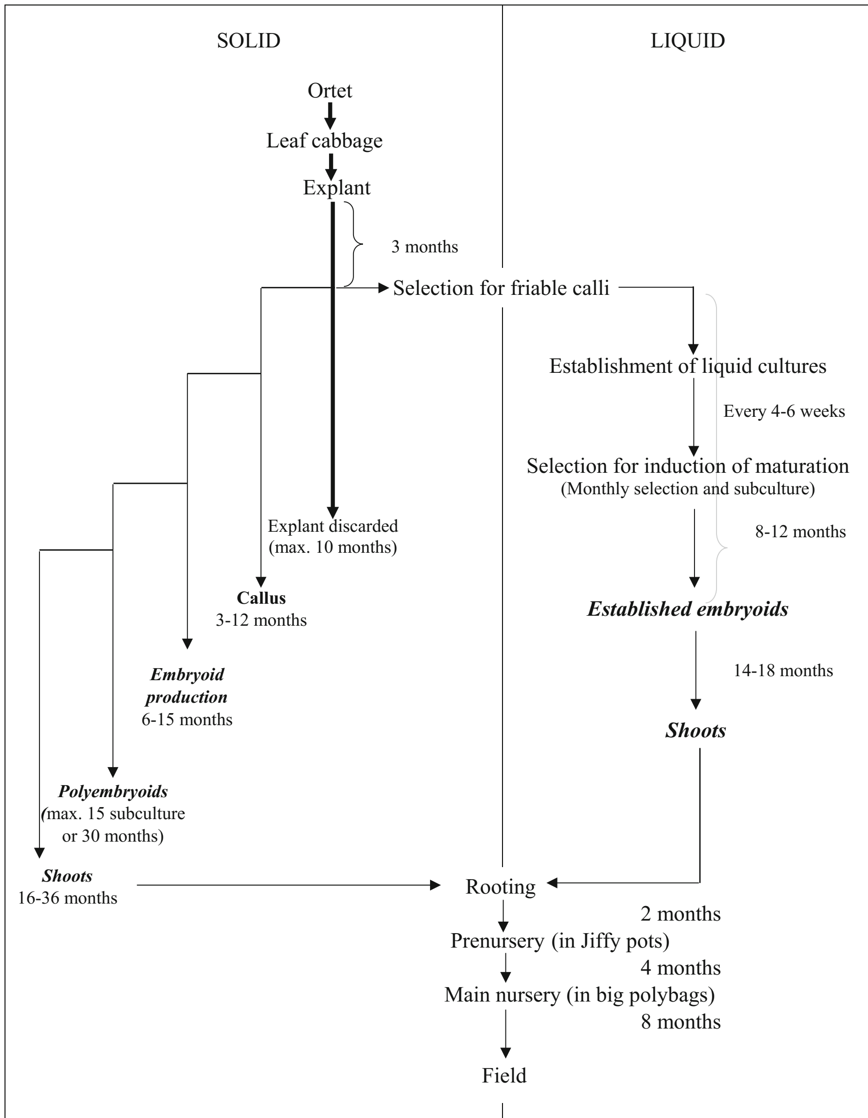


Fig. 18.9 MPOB protocol with time scale

18.7.2 Quality Control

Physical control (Culling)

Culling is carried out at both the in vitro and ex vitro stages. At the in vitro stage, callus cultures that appear flaccid and brownish are discarded. It is quite common to obtain various types of embryoids. They can be nodular, torpedo-shaped (single or fused), globular, wavy, etc. It is not known whether the different morphotypes will give rise to abnormality. From our experience, the nodular, torpedo-shaped (fused) and wavy types are generally more prolific. Most of the culling is carried out at the shoot and rooting stages. A normal DxP seedling derived from the embryo rescue method is used as a reference. Stunted erect shoots and shoots producing terminal inflorescences are discarded. Shoots with fleshy bases and poorly developed laminae are also discarded (Tarmizi 1997; Rohani et al. 2000; Ong-Abdullah et al. 2005). At the ex vitro stage (pre-nursery), abnormal shoots tend to be stunted, remain juvenile for prolonged periods and show nutrient deficiency or diseased symptoms. Such ramets are discarded.

Molecular control (*SureSawit*™ KARMA)

The mantling abnormality affects fruit development and thus oil yields from clonal palms. Although the mantling rate has been reported to be at an average of 5%, it varies widely and unpredictably depending on genotypes or culture conditions (Jaligot et al. 2011). A diagnostic marker to screen clonal planting material at an early age would be favorable to reduce the risk of field planting abnormal plants. This was made possible by the recent discovery that hypomethylation of a *Karma* retrotransposon at the MANTLED locus was associated with this abnormal phenotype (Ong-Abdullah et al. 2015). A diagnostic assay was subsequently developed for screening palms using leaf samples.

The *SureSawit*™ KARMA assay is a PCR-based method to detect the level of methylation at the Karma region (Ong-Abdullah et al. 2016a, b). A leaf punch measuring 0.5 cm in diameter is sampled using the proprietary leaf puncher in the kit. DNA is then extracted from the sample through an automated process in the laboratory. Bisulfite conversion followed by desulfonation is then carried out on the DNA to convert unmethylated cytosines to uracil bases, while methylated cytosines on the DNA will remain unconverted. After having gone through several rounds of optimization, the final converted DNA, which is used as the template undergoes subsequent probe-based PCR assay. Various controls are incorporated into the analysis to determine the methylation levels of the target site in the DNA and finally, predict the mantling risk of the sampled plant (Figs. 18.10 and 18.11). This diagnostic test can be utilized as a decision making tool for the industry. Tissue culture laboratories can then plan and refine their cloning strategies and subsequent clonal plantings.

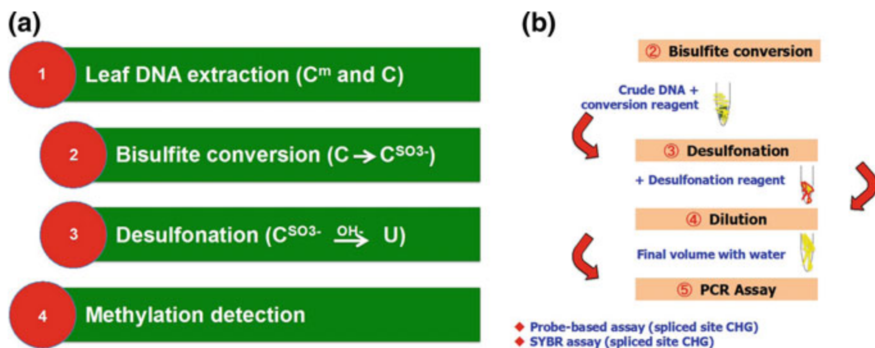
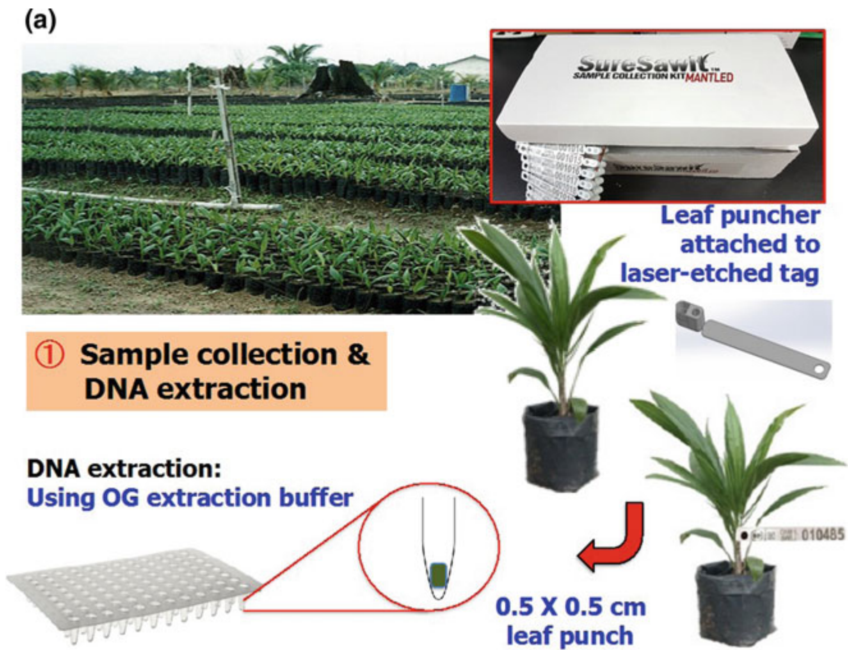


Fig. 18.10 **a** The general process flow of the KARMA assay and **b** Steps involved in preparation of DNA for the PCR assay

18.8 Conclusion and Future Technologies

To ensure a successful venture into the production of clonal oil palm materials, a good established breeding programme is essential for the identification of superior palms or ortets. In relations to this, MPOB, having the largest oil palm germplasm collection in the world, has continuously evaluated its collection for traits of economic interest and value (Mohd Din et al. 2005). Acknowledging the fact that conventional breeding is time consuming, tissue culture has now become an appropriate strategy for expediting the development of commercial planting materials. However, requirement of a high number of ortets for tissue culture remains a setback.

The liquid culture system was demonstrated to have significant effects on multiplication rates, morphology of shoots, somatic embryo development, microtubers or bulblets produced in vitro (Preil 2005). Liquid cultures were also proven to be a good source of starting material for transformation and protoplast isolation studies (Xiao et al. 2009; Sallets et al. 2015) as well as for secondary metabolite production (Zare et al. 2010; Boonsnongcheep et al. 2010). However, the productivity of cultures in the conventional liquid culturing system is limited due to the size of vessels used. Various innovative technologies have been developed to further improve the efficiency of the liquid culture system. Technologies such as the MPOB Fast Transfer Technique (MoFaTT) (Tarmizi and Zaiton 2007) in liquid culture system, the 2-in-1 MoSlim (MPOB Simple Impeller for liquid culture, the Simple Impeller with Fast Transfer Technique (SLIM-FaTT), the MPOB Modified Vessel (MoVess), MultiVessel (MV) bioreactor, Motorized Vessel (MotoVess) and Motorized Vessel with Fast Media Transfer (MoVeFast) (Tarmizi et al. 2009, 2016) systems allow rapid and convenient propagation of liquid cultures at a larger scale compared to the conventional shake flasks system. These innovations can be applied to any fluidic platforms even in other crops and can be a catalyst for future semi- or fully-automated liquid culture systems.



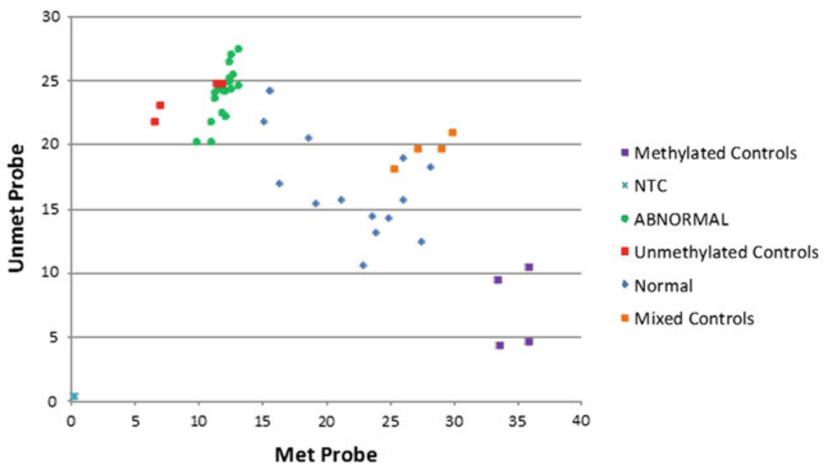


Fig. 18.11 **a** Sampling procedure made-easy with the SureSawit^{TM} KARMA collection kit and OG DNA extraction buffer and **b** Methylation detection Adapted from Ong-Abdullah (2016)

Due to the slow process of oil palm in vitro propagation, which takes two to five years from the start of the explant stage to acquiring nursery ramets, oil palm clones cannot meet the entire demand for improved planting materials in the near future. However, while moving ahead with the production of clones, high-performing duras and pisiferas with good combining abilities are also cloned for use in the mass production of "clonal seeds". Clonal seeds can be in the form of either a semi-clone, whereby only one of the parents is cloned (often is the dura) or a bi-clone, when both parents are tissue culture-derived. One of the setbacks in tissue culture is the high number of ortets required to meet production demands. Therefore, the proposed establishment of ortet gardens would certainly aid in overcoming the limited source of explants.

Essentially, Malaysia being limited in arable land is not in the position to stay competitive based on planted area. Therefore maximizing productivity through increasing oil yield by utilizing the best planting materials and possibly reconfiguring estates should be emphasized. In parallel, policies and regulations may need to be re-examined. This requires close cooperation between the various agencies and strengthening the support mechanisms towards realizing the aspirations set by the government.

Clonal planting material is expected to create the "second wave" in yield improvement. Clones tested with the *SureSawit*™ KARMA assay will assist in minimizing mantling to further boost yield productivity. With tissue culture labs imposing higher than the minimum selection standards and coupled with implementing Good Agricultural Practices in plantations, oil yield is expected to substantially increase the overall national productivity.

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References

Aziah MY (1989) Shoot formation in *Calamus manan* under *in vitro.* In: Rao AN, Aziah MY (eds) Proceedings of the seminar on tissue culture of forest species, June 15–18 1987. Kuala Lumpur, Malaysia, pp 45–49

Baudouin L, Durand-Gasselin T (1991) Genetic transmission of characters linked to oil yields in oil palm by cloning results for young palms (1). Oléagineux 49:313–320

Boonsnongcheep PP, Korsangruang SS, Soonthornchareonnon NN, Chintapakorn Y, Saralamp P, Prathanturarugand PS (2010) Growth and isoflavonoid accumulation of *Pueraria candollei var. candollei* and *P. candollei var. mirifica* cell suspension cultures. Plant Cell Tissue Organ Cult 101:119–126

Corley RHV, Gray BS (1976) Yield and yield components. In: Corley RHV, Hardon JJ, Wood BJ (eds) Oil Palm Research. Elsevier, Amsterdam, pp 77–86

Corley RHV, Lee CH, Law IH, Wong CY (1986) Abnormal flower development in oil palm clones. The Planter Kuala Lumpur 62:233–240

Duval Y, Durand-Gasselin T, Konan K, Pannetier C (1988) *In vitro* vegetative micropropagation of oil palm *(Elaeis guineensis* Jacq.) strategy and results. In: Proceedings of the 1987 international oil palm/palm oil conference—agriculture, June 23-July 1 1987, PORIM, Bangi, pp 191–198

Eeuwens CJ, Blake J (1977) Culture of coconut and date palm tissue with a view to vegetative propagation. Acta Hort 78:277–281

Jaligot E, Adler S, Debladis E, Beule T, Richaud F, Ilbert P, Finnegan EJ, Rival A (2011) Epigenetic imbalance and the floral developmental abnormality of the in vitro-regenerated oil palm Elaeis guineensis. Ann Bot 108:1453–1462

Jones LH (1974) Propagation of clonal palms by tissue culture. Oil Palm News. 17:1–8

Kushairi A, Rajanaidu N (2000) Breeding populations, seed production and nursery management. In: Yusof B, Jalani BS, Chan KW (eds) Advances in oil palm research, vol 1. MPOB, Bangi. pp 39–96

Kushairi A, Mohd Din A, Rajanaidu N (2011) Forging ahead with clones In: Wahid MB, Choo YM, Chan KW (eds) Further advances in oil palm research (2000–2010), vol 1. MPOB, pp 47–101

Mohd Din A, Rajanaidu N, Kushairi A (2005) Exploitation of genetic variability in oil palm. IN: Proceedings MOSTA best practices workshops: agronomy and crops management, Malaysian Oil Sci Technol Assoc., March 27-August 14. Malaysia, pp 19–42

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio-assays with tobacco tissue cultures. Physiol Plant 15:473–497

Ong-Abdullah M, Rohani O, Zamzuri I, Tarmizi AH, Sharifah SA (2005) Handbook: photographic perspective on the anomalies of oil palm. Published by the Malaysian Palm Oil Board

Ong-Abdullah M, Ordway JM, Jiang N, Ooi S-E, Kok S-Y, Sarpan N, Azimi N, Hashim AT, Ishak Z, Rosli SK, Malike FA, Abu Bakar NZ, Marjuni M, Abdullah N, Yaakub Z, Amiruddin MD, Nookiah R, Singh R, Low E-TL, Chan K-L, Azizi N, Smith SW, Bacher B, Budiman MA, Van Brunt A, Wischmeyer C, Beil M, Hogan M, Lakey N, Lim C-C, Arulandoo X, Wong C-K, Choo C-N, Wong W-C, Kwan Y-Y, Syed Alwee SSR, Sambanthamurthi R, Martienssen RA (2015) Loss of Karma transposon methylation underlies the mantled somaclonal variant of oil palm. Nature 525:533–537

Ong-Abdullah M, Ordway JM, Jiang N, Ooi S-E, Mokri A, Nookiah R, Singh R, Low E-TL, Sachdeva M, Smith SW, Lakey N, Sambanthamurthi R, Martienssen RA (2016a) Tissue culture and epigenetics. The Planter. Kuala Lumpur. 92:741–749

Ong-Abdullah M, Ordway JM, Jiang N, Ooi S-E, Mokri A, Kok SY, Sarpan N, Azimi N, Hashim AT, Ishak Z, Rosli SK, Nookiah R, Singh R, Low E-TL, Sachdeva M, Smith SW, Lakey N, Martienssen RA, Sambanthamurthi R (2016b) *SureSawit*™ KARMA—a diagnostic assay for clonal conformity. MPOB Information Series No. 156, Malaysia

Paranjothy K, Othman R (1982) *In vitro* propagation of oil palm. In: Fujiwara A (ed) proceedings of the fifth congress plant tissue and cell cult. July 11–16 1982, Maruzen, Tokyo. pp 747–748

Preil W (2005) General introduction: a personal reflection on the use of liquid media for *in vitro* culture. In: Hvoslef-Eide AK, Preil W (eds) Liquid culture systems for *in vitro* plant propagation. Springer, Berlin, pp 1–18

Rabéchault H, Martin J-P (1976) Multiplication vegetative du palmier a huile *(Elaeis guineensis* Jacq.) al'aide de cultures de tissus foliares. C.R Acad Sci Paris 283:1735–1737

Rabechault H, Ahee J, Guenin G (1970) Colonies cellulaires et formes embryos *in vitro* á partir de cultures d' embryons de palmier a huile (*Elaeis guineensis* Jacq. Var. dura Becc.). C.R. Acad Sci Paris 270:3067–3070

Rajanaidu N, Rohani O, Jalani S (1997) Oil Palm clones: current status and prospects for commercial production. Planter 73(853):163–184

Rohani O (2001) Positional effect of leaf explant on callusing and embryogenesis in Oil Palm *(Elaeis guineensis).* In: Proceedings of the 2001 PIPOC international palm oil congress—agriculture, MPOB, Bangi, pp 677–685

Rohani O, Sharifah SA, Mohd Rafii Y, Ong M, Tarmizi AH, Zamzuri I (2000) Tissue culture of oil palm. In: Yusof B, Jalani BS, Chan KW (eds) Chapter 7, advances in oil palm research, vol 1. MPOB, Bangi. pp 238–283

Sallets AA, Delimoy D, Boutry M (2015) Stable and transient transformation of *Artemisia annua* suspension cells. Plant Cell Tissue Organ Cult 120:157–164

Smith WK, Thomas JA (1973) The isolation and *in vitro* cultivation of cells of *Elaeis guineensis*. Oleagineux 28:123–127

Sogeke AK (1998) Stages in the vegetative propagation of Oil Palm, *Elaeis guineensis* Jacq. through tissue culture. J Oil Palm Res 10(2):1–9

Tarmizi AH (1997) Assessment of variability in tissue culture-derived buds of Oil Palm (*Elaeis guineensis* Jacq.). Ph.D. thesis. University of Liverpool, England, 234 pp

Tarmizi AH, Zaiton R (2007) Development of the MPOB Fast Transfer Technique (MoFaTT) for maintenance and maturation of oil palm culture aggregates. J Oil Palm Res. 19(2):435–439

Tarmizi AH, Zaiton R, Rosli MY (2009) MPOB Motorized Vessel (MPOB-MotoVess) for liquid tissue culture system. MPOB Information Series TT No. 454

Tarmizi AH, Zaiton R, Rosli MY (2016) Simple impeller system for maintenance of Oil Palm culture aggregates. J Oil Palm Res 1928(2):184–190

Thanh-Tuyen NT, Apurillo DI (1992) Plant regeneration through somatic embryogenesis and cultured zygotic embryos of coconut. Pakistan J Contemp Sci 17(1):1–7

Tisserat B, Foster G, de Mason D (1979) Plantlet production *in vitro* from *Phoenix dactylifera L.* Date Growers' Institute Rep 54:19–23

Wong G, Tan CC, Soh AC (1996) Large-scale propagation of oil palm clones - experience to date. Paper presented at the International symposium on *in vitro* culture and horticultural breeding, June 16-21 1996. Jerusalem, Israel. pp 649–658

Xiao W, Huang X, Gong QDX, Zhao JT, Wei YR, Huang XL (2009) Somatic hybrids obtained by asymmetric protoplast fusion between Musa Silk cv. Guoshanxiang (AAB) and Musa acuminate cv. Mas (AA). Plant Cell Tissue Organ Cult 97:313–32

Zamzuri I (1998) Efficient rooting of Oil Palm *in vitro* plantlets using double-layer technique. PORIM Bulletin No. 36:23–36

Zamzuri I (1999) Using double-layer technique in rooting of Oil Palm *in vitro* plantlets. In: Proceedings of the 1999 PORIM international palm oil congress-agriculture, February 1–6 1999. MPOB, Bangi. pp 223–229

Zamzuri I (2004) Clonal trial report. Biology Division, MPOB. 26-3-2004