Chapter

An Overview of Oil Palm Cultivation via Tissue Culture Technique

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Abstract

During the last three decades, plant cell, tissue, and organ culture have developed rapidly and become a major biotechnology tool in agriculture, horticulture, forestry, and industry. Many problems in conventional breeding techniques were solved via tissue culture techniques. Plant tissue culture technique permits the growing plants in test tube or closed container in vitro under controlled environment. This technique is devoted to solve two problems: 1) To keep the plant cells free from microbes. 2) To grow the desired plants by providing suitable nutrient medium and other environmental conditions. In this chapter, a review around plant tissue culture techniques that have been reported on oil palm breeding programme will be discussed. It is including the laboratory techniques, advantages and disadvantages of the technique, the problems to produce good and prolific oil palm tissue culture clones and mitigation measures that have been reported to overcome the problems. As a conclusion, this chapter reviews tissue culture techniques that could be used to propagate oil palm clones.

Keywords: oil palm, propagation, tissue culture, overview

1. Introduction

Plant tissue culture is a technique used to propagate plants in vitro (in test tube) under sterile conditions, often to produce clones of a plant. During the last three decades, plant cell, tissue, and organ culture have developed rapidly and become a major biotechnology tool in agriculture, horticulture, forestry, and industry. Many problems in conventional breeding techniques were solved via tissue culture techniques.

In nature, there are a variety forms of plants either seed plants for example, trees, herbs, grasses, or flowering plants for example, fruit-bearing trees. Plants exhibit the basic morphological for example, root, stem and leaves. However, they are vary with differences in cells and tissues, and their topography.

In tissue culture, the term 'culture' refers to the fragments of plants that are grown in nutrient media. There are several types of cultures, which are cell culture, tissue culture, organ culture, explant culture, and protoplast culture. Explant is the excised fragment of plants that is transferred into nutrient media. There are many types of explants, it can be roots, stems, leaves, seeds, fruits, and flowers.

The role of explant is to initiate culture in a nutrient media, provided that they must be able to de-differentiate into totipotent cells.

The term 'Tissue Culture' or micropropagation is the technique to propagate plants under sterile conditions, often to produce clones of a plant. The cultured of explant on a solid medium produces mass of protoplasmic cells which later be induced to become a complete plant. However, in organ culture, for example, excised roots, the cultured explant (plant material) maintains its morphology identity, more or less, similar with the same physiology as in vitro of the parent plants. Plant organs are referred to part of plants that possess vascular tissues such as root, shoot, and leaves. Embryo is an independent structure and does not have vascular supply, thus, embryo is not supposed to be the plant organ.

2. What is totipotency?

Totipotency is the unique feature of plant cells where every single cells has biological potential to develop normal root, shoots and embryoids, which ultimately forming a plant. The term totipotency is defined as the capability of each living cell to carry out independent development to regenerate into a complete plant, provided with suitable conditions [1]. Many research has been done using tissue culture as a medium to study multicellular organism, because it is rather difficult when using the organism as biological units of study.

3. Problems in oil palm tissue culture

The oil palm clones are frequently generated via indirect somatic embryogenesis, in which the plantlets were produced from the growth of callus and differentiate into somatic embryos. The process is usually induced from seeds, hence, making seed propagation most common for oil palm somatic embryogenesis. However, seeds propagation often cause difficulties in terms of low germination of the seeds. Due to the hard woody and sturdy structure of oil palm, there is very few choices of explants that can be used to induce somatic embryogenesis. Apart from the seeds, immature leaves are another explant of oil palm that is often be opted.

Oil palm is one of recalcitrant plants, meaning that its explants are more difficult to be developed into plantlets as compared to other plant species. While somatic embryogenesis is well known for its low efficiencies in producing small number of plantlets, the other main setbacks of this method in oil palm breeding are the difficulties in initiating somatic embryogenesis out of embryo cell cultures as well as to fully convert those somatic embryos into whole plantlets. It was reported that immature leaves explants of oil palm has been able to generate callus up to 52%, while other explants such as zygotic embryos and immature inflorescence produced higher rate of callus induction [2]. Despite of these reported adequate efficiencies, the callus induction rate from an explants are highly dependent of its genotype. Many explants could only regain totipotency during the culture process at around 1–5% in order to initiate callus, thus, making somatic embryogenesis is very inefficient procedures [3]. The development of somatic embryogenesis into whole plantlets is also often problematic. This is due to the failure of shoot or root development or induction, whereby, results in low regeneration rate of oil palm clones [4, 5].

The combination of low efficiency of somatic embryogenesis induction, limited explants choices, and low regeneration rate into complete explants, making oil palm breeding via tissue culture is impossible without embryos proliferation process is carried out. However, by producing large number of embryos in one culture may

produce risk of somaclonal variation. Another way to increase regeneration rate in oil palm culture is by using explant of prolific breed. Although the selection of this breed requires large screening process at molecular level to identify the best genotype, it could be developed as a tool that possibly provide long-term benefits [6, 7].

4. Regeneration of cultured plants

4.1 Organogenesis

There are several methods to regenerate cultured plants, one of it can be through organogenesis, either through direct organogenesis or indirect organogenesis. Organogenesis is the development of individual plant organ such as shoots or roots from the cells in culture (can be callus (indirect) or plant tissue (direct)) by the process of differentiation. Organogenesis in plant tissue culture involves two stages: Dedifferentiation and redifferentiation. Dedifferentiation starts shortly after explant initiated rapid cell division and consequently forms a mass of undifferentiated cells (callus). Redifferentiation, also referred as budding, is the process where the callus starts to differentiate to form plant organ (organ primordia). This primordia organ is then develop into small meristems (which contains of large nuclei).

In direct organogenesis, the formation of plant organs such as shoot and root is straight from organized tissue (explant) without undergoes callus formation. Progenies that are produced through this technique have identical genetic content as parent. However, this technique is depending on several factors such as explant type, age of explant and size of explant. If meristem tissues is used as explant, the genetic content of the progeny (offspring) are identical as parent. Conversely, if embryos are used as explant, the genetic content between progeny and parent are not identical as embryo formed through fertilization of gamete cells (male and female gametes) and some plants have dormancy period.

In indirect organogenesis, callus is produced first from explant. Callus is disorganized group of cells, rapid dividing, and undifferentiated into specialized tissues such as shoots and roots. Callus can be induced from callus culture, explant (tissues) or cell suspension culture of that callus. Subsequently, organ formation is induced from the callus where shoots and roots are stimulated by plant growth hormones. However, the disadvantage is the changes or variation in the genetic content of somatic cells of the progeny (somaclonal variation) due to repetitive subcultures. Direct organogenesis can be opted to avoid somaclonal variation. Direct organogenesis is often used to plants that are difficult to propagate and do not have abundance of meristem tissues. Therefore, these plants are propagated by using leaves, stems, and root tips as explants.

The rule of thumbs in organogenesis technique are the proportion of growth hormones combinations in the culture medium used to stimulate the respective organs. In direct organogenesis, high ratio of auxin to cytokinin will produce roots while high ratio cytokinin to auxin will induce shoots. In indirect organogenesis, balance or same ratio of auxin and cytokinin (1:1) will produce callus.

Organogenesis starts with induction process caused by the plant hormones in the medium, substances carried over from the explants and endogenous hormones produced by the explants. Organogenesis was first induced by Skoog in 1944 on the formation of root and inhibition of shoot after the addition of auxin. It was then proposed that the regulation of organogenesis is depending on the balance between auxin and cytokinin. The research team then later discovered that high ratio of auxin to cytokinin stimulated the root formation in tobacco callus, but a low ratio of auxin to cytokinin led to shoot formation (**Table 1**).

Auxin (mg/l)	Cytokinin (mg/l)	Organogenesis
0.0	0.2	No growth
0.03	1.0	Shoots
3.0	0.02	Roots
3.0	0.2	Callus

Table 1.
Standard concentrations of auxins and cytokinins to induce in vitro *organogenesis.*

4.2 Somatic embryogenesis

Other than organogenesis, somatic embryogenesis is another major regeneration technique in plant tissue culture. Embryo production is an important feature of the flowering plants. The process of embryo formation is called embryogenesis which starts from a single embryogenic cell and subsequently develops into either a zygote or undifferentiated callus cells. Embryo that develops from zygotes is called as zygotic embryos. Meanwhile, embryo that develops from somatic cells is called as somatic embryos where it is artificially induced in cultured plant tissues.

Somatic embryogenesis was first induced in cell suspension culture and callus culture of carrot. Other plants like *Coffea Arabica*, *Citrus cincensis*, *Nicotiana tabacum*, *Pinus ponderosa* and *Cocos nucifera* are among successful species in somatic embryogenesis. In plant tissue culture, somatic embryogenesis is the formation of somatic embryoids from somatic tissues of callus or cells of suspension culture, which can then develop into complete plants in a similar way to the zygotic embryos (sexual reproduction). Somatic embryoid (asexual embryo) is small and well-organized structure that is resemblance to zygotic embryo (sexual embryo), which is produced from embryogenic somatic cells. Somatic and zygotic embryoids share the same pattern of development where both undergo globular, heart, torpedo shaped and cotyledon stage for dicots and conifers. Embryo growth is bipolar which produces a shoot and a radicular pole at the other end. When encapsulated with suitable nutrient, somatic embryos become artificial or synthetic seeds and they as they can produce plantlets and planted directly into the field.

Somatic embryos can be produced through direct or indirect somatic embryogenesis. In direct somatic embryogenesis, the embryo is induced directly from cells or tissues without the formation of intervening callus. However, this technique is rare and uncommon compared to indirect somatic embryogenesis. In indirect somatic embryogenesis, callus if first formed from explant. Somatic embryos can be then induced from the callus or cell suspension culture of that callus. The embryoids are initiated from superficial callus aggregates where the cells contain large vacuole, dense cytoplasm, large starch granules and nucleus.

Two types of medium with different compositions are required to induce somatic embryoids. First medium contains auxin to initiate embryogenic cells. Second medium is lacked or reduced of auxin, is needed to support the development of the embryogenic cells into embryoids and plantlets. Similar to zygotic embryos, the embryogenic cells pass through 3 different stages i.e. globular, heart shaped, and torpedo shaped, to form embryoids. The embryoids can be separated from the non-embryoids callus cells by using glassbeads or filter paper.

The importance of somatic embryogenesis in agriculture, horticulture, and plant conservation is the zygotic and nucellar embryogenic can be obtained separately from the polyembrogenic plants such as citrus. Since somatic embryo has no food reserves, they can be preserved as encapsulated seeds (surrounded with

Zygotic embryo	Somatic embryo
Fertilized egg or zygote	Somatic cells
Contain seed coat	No seed coat
Produce seed	Produce embryo only
Plantlets are healthy	Plantlets are weaker
Not indentical to mother plant	Identical to mother plant
Propagation rate is low	Propagation rate is high

Table 2.
Comparison between zygotic and somatic embryo.

nutrients). This makes international exchange of germplasm possible. This artificial seeds provide an advantage for embryos of big and heavy fruits like coconut which can be preserved in a test tube for months and then cultured on medium. In addition, some plants that are crossed interspecific or intergeneric are failed to develop at maturity stage, therefore, before the embryos undergo maturity, they can be taken and cultured on artificial medium and grown into whole plants. As somatic embryogenesis produces many somatic embryos in cell culture, this technique is regarded as the ideal mass propagation system. The somatic embryo is a bipolar system which can develop directly into complete plant, hence, there is no need for separate rooting and shooting induction steps. Plants that derived from somatic embryo may be free of viral and pathogens. Therefore, it is another option in disease-free plants generation (**Table 2**).

4.3 Somaclonal variation

Somaclonal variation produces phenotypic variation in the somaclones either through genetic variation or epigenetic. In oil palm tissue culture where it is propagated through somatic embryogenesis, this technique is often lead to somaclonal variation [3]. Somaclonal variation refers to genetic variability generated during tissue culture and can be detected as genetic or phenotypic traits. Several features to identify somaclonal variation in somaclones are by examining the number and structure of chromosomes. Somaclones with altered chromosomes are usually exhibit changes in leaf shape and color, growth rate and sexual fertility. It is a heritable mutations and persist in next generations even after plantation into the field.

Somaclonal variation can be developed in tissue culture through genetic and epigenetic mechanisms. In genetic mechanism, variations are presence in somatic cells of explant which may be caused by DNA changes and mutations. In epigenetic mechanism, somaclonal variation is generated during tissue culture and results in temporary phenotypic changes. Somaclonal variation can also occurs due to physiological effect such as exposure to plant growth hormones and the culture conditions.

4.4 Screening work-flows for somaclonal variants

There are various methods to select somaclonal variants.

i. Analysis of morphological traits.

- Qualitative: Plant height, maturity date, flowering date, and leaf size
- Quantitative: Number of flowers, leaves, and seeds.

ii. Cytological studies

Feulgen staining can be used to stain nuclei of the somaclones to measure DNA contents by using cytophotometer. Number of chromosomes can also be measured using the same technique on meristematic tissues such as root tip and shoot tip.

iii. Gel electrophoresis

This technique can be used to detect variation among somaclones in terms of the concentration of protein, pigments and amino acids through the observation of amplification pattern.

iv. Disease resistance trait

Pathogen or toxin that is responsible for the disease resistance can be used to select disease resistance clones.

v. Herbicide resistance trait

Plantlets that are grown on culture medium containing the particular herbicide possess the herbicide resistance trait.

vi. Stress tolerance trait

Detection for stress tolerance trait has been done on tobacco cell lines for high salt tolerance, and drought tolerance in tomato.

4.5 Advantages of somaclonal variations

Somaclonal variations help in crop improvement. In India, a somaclonal variant *Citronella java*, a medicinal plant that has been named as "Bio-13", produce yield increment by 37% oil content and 39% more citronellon (type of terpenoid that can be used in skin products) than the control plant. In US, a new somaclonal variant was developed and called as "Supertomatoes" where these clones capable in reducing shipping and processing costs. Other advantages of this technique are as following:

- Allow the generation of genetic variations
- Increased and improved production of secondary metabolites
- Enable the generation of plants resistant to toxins, pesticides, herbicides and plants that tolerant to stress (unfavorable condition) such as drought and high salt concentration.
- Suitable for breeding of tree species

4.6 Disadvantages of somaclonal variations

The main setback that has made this breeding technique difficult to carry out is the difficulties to obtain a uniform clones as this feature is important in mass

propagation of the plants especially in horticulture and forestry industries. In order to select uniform clones, extensive and extended field trials may be required. The genetic variations in somaclonal variant plants are also unstable, thus, the desired traits have tendency to disappear in the next generation. There is also risk where the clones may exhibit undesirable traits.

5. Types of explant and explant selection

Explant is a sterile excised fragment of plants from which cultures are initiated. Generally, all types of plant cells or tissues can be used as an explant, however, it is preferable to use young and immature tissues that is rapidly dividing (at early stage of development) as an explant such as shoot tip, root tip, and young leaves. There are several factors need to be considered during explant selection process:

i. Explant age – Physiologically, younger tissues are more responsive to the medium in order to induce cell division. Younger tissues are also more suitable as explant because the tissues surface are softer and that helps to ease sterilization process whereby, helps in preventing contamination.

ii. Explant size - The smaller the explant is the better to minimize possibilities of contamination from bacteria, fungus, and viruses. However, small sized explant has lower survival rate in the medium as compared to larger explant due to the lack of reserve nutrient available to sustain the culture.

iii. Season – The season of the year may have effects on explants survival rate in the medium and contaminations. For example, shoots or leaves that are taken during the Spring season are more responsive compared to other seasons. In Malaysia, which is a tropical country, this factor may not need to be considered.

iv. Plant quality – It is more suitable to select explant from plants that are healthy rather than plants that are under stress conditions such as water-stress or nutritional stress or plants that exhibit disease symptoms.

Apart from the above factors to select the best explant, another thing that is need to consider in selecting an explant is the goal of the experiment. The choice of explant tissues is depending on what type of response desired from the cell culture. For example, if the aim is to carry out clonal propagation, explant of shoot or root tip is suitable to achieve it. For callus induction, fragment of cotyledon, hypocotyl, stem, leaf, and embryo can be used as explant. For protoplast fusion, leaf tissue from aseptically germinated seeds are preferable.

6. The advantages and limitations in tissue culture technique

Plant tissue culture technique permits the growing plants in test tube or closed container in vitro under controlled environment. This technique is devoted to solve two problems: 1) To keep the plant cells free from microbes. 2) To grow the desired plants by providing suitable nutrient medium and other environmental conditions. Several advantages of plant tissue culture techniques are as following:

i. Uniform growth – As tissue culture plants (clones) possess the same genetic content, the progenies undergo the same growth pattern.

ii. True to type – Tissue culture plants are grown asexually from somatic cells of mother plant (explant). Therefore, the progenies possess the same genetic content as the explant.

iii. Increase availability of plants – Some plants are difficult to grow. Tissue culture provides the solution to multiply plants in large scales and in uniform growth. Industries that uses plants as raw materials such as in pharmaceutical or medicinal products require large quantity of plants, which can be produced by using tissue culture.

iv. Continuous supply of plants – Some diseases are fluctuate depending on changing of seasons, climate and crop diseases. Therefore, there is a need for continuous supplies of the medicinal plants for production of drugs, that can not be synthesized synthetically. Novel plant – Tissue culture facilitates in developing novel plants with desired traits that are generated through gene transformation.

v. Disease free and desired propagule – Plants can be grown in disease free environment in large scale, and the desired propagule such as buds and stems can be transported to other places without any damage.

vi. Biosynthetic pathways – Tissue culture can be used for detecting the production of secondary metabolites using labeled precursor in the medium. For example, the production of anthocyanin in apple callus culture.

vii. Immobilization – Tissue culture technique can be used for plant preservation. Through the production of artificial seeds, it allows the immobilization (entrapment) of tissue whereby minimize transportation handling and cost.

viii. Continuous production of medicinal drugs – Some medicinal plants are may be seasonal, and are difficult to obtain due to climate change, etc. Tissue culture acts as a tool to allow the production of the natural compounds independent of soil, climate change and seasons.

ix. Seedless propagation – Some plant seeds are difficult to germinate, tissue culture enables plant propagation without the need of seeds.

6.1 Limitations in plant tissue culture

Despite of various advantages of plant tissue culture, this technique has some limitations.

i. High level of expertise are required – Tissue culture technique require excellent level of handling skills as a small error can lead to damage of products or plants. Companies or institutions need to invest on staff training and that makes it costly, as it requires a long-term capital investment.

ii. Expensive – Other than high cost in expertise, tissue culture is also require high cost in chemicals which must contains in high purity.

iii. Low production of secondary metabolites – Amount of secondary metabolites that are harvested from tissue culture plants are often negligible. Therefore, it requires large scale production of plants in order to increase yield.

iv. Instability – Despite having an advantage of producing clones that are identical to the explant, there are times where genetic variation occurs among clones lines which cause changes on phenotype and genotype of the clones. This is called as somaclonal variation.

v. Prone to contamination – Aseptic technique are need to maintained throughout the *in vitro* plant growth. If contamination occurs, the plant growth is impaired and has low survival rate.

7. Shoot culture and micropropagation

In shoot culture, apical meristem (located at shoot apex and root apex) is cultured, and this culture is also known as meristem culture due to large size of the explant (5–10 mm). Shoot culture is widely applied in horticulture, agriculture and forestry. Murashige, from Morel research team (1960), has significantly established the technique for micropropagation and its further biotechnological application. Due to the small size of explant to be propagated and it occurs *in vitro* as compared to conventional propagation, this technique is also known as micropropagation. Stages in micropropagation are as following:

Stage I Selection and preparation of explant – Suitable explant is selected and inoculated into nutrient medium. This step is done is extreme aseptic condition.

Stage II Multiplication of cells – Growth of culture takes up to 2 months, followed by repeated subcultures.

Stage III Shooting and rooting induction – Culture is transferred to nutrient medium with suitable composition to induce multiple shoots. This step may take about 4 weeks. Subsequently, the culture is transferred on medium suitable for root induction and incubated for about 3 weeks.

Stage IV Transfer to soil – After about one month of culture, plantlets are aseptically removed from test tube environment to natural and harsh environment. At this stage, roots should be fully functional in potting soil mix. During this step of transplantation, plantlets have tendency to fail to survive due to desiccation i.e. from 100% humidity of test tubes to low humidity under ambient conditions), unfavorable environment, invasion of soil microorganisms, as well as fails to adapt changes from dependent (artificial medium) to independent environment.

8. Mass propagation

8.1 Acclimatization

In order to minimize the failure of transplantation, it is necessary to develop acclimatization capability in plantlets before transfer them into soil. Acclimatization is the process where an organism adapts to changes in its environment such as change in temperature, humidity, photoperiod, and pH, to allow it to survive in a range of environmental conditions. This can be done by:

i. Induction to develop some normal and functional leaves

ii. Induction of functional roots

iii. Exposing the *in vitro* cultures to harsh environment in two weeks before planting out.

8.2 Plantlets transplantation from in vitro culture to polybags

The following procedures are standard practice for transplantation:

i. Plantlet is removed from test tube and washed under running tap water.

ii. Soil mixture is prepared with ratio 3:2:1 of soil, peat soil, and fine soil.

iii. Polybags and small planting pots (8 cm x 8 cm) are prepared.

iv. Cocoa peat soil is filled into the base of polibags or planting pots.

v. The soil mixture is then added into the polybags and planting pots.

vi. Roots of plantlets are dipped into IBA rooting powder.

vii. The plantlets are planted into the soil mixture until the root area is covered.

viii. The plantlets are watered and then covered with transparent plastic, with holes on the surface to allow gas exchange.

ix. The plantlets are placed under up to 60% shading area, and strictly monitored in nursery for 2–3 months.

x. The plantlets are watered using mist sprayer and the plastic cover is removed after 1 month of planting.

xi. After 3 months, the plantlets are removed to bigger size of polybags or planting pots with the same soil mix composition.

xii. The plantlets are continuously watered and monitored until 4 months old before it is transferred to the orchard.

8.3 Transfer of plantlets to orchard (mass propagation)

After acclimatization, the plantlet is ready to be transferred to orchard in larger planting area. This step is called mass propagation.

i. After 3 months old of the plantlet, the shading is reduced to 20%.

ii. The plantlet is fed with NPK fertilizer 20:20:20 (Nitrogen:Phosphorous:Kalium). The fertilizer is diluted beforehand with water, to reach the concentration between 150 ppm to 200 ppm.

iii. After the plantlet reach 4 months old, it is then ready to be transferred to orchard.

iv. The planting holes are digged beforehand. The depth is depending on the plantlet size, to cover all the root system into the soil.

v. Mixture of organic fertilizers per plantlet can be added into the planting hole such as chicken or cows manure (100 g), triple superphosphate (100 g), and lime fertilizer (calcium carbonate) (100 g).

vi. Plantlet is removed from polybag and planted into the hole. The fertilizer mixture is added until the top of the soil and covers the area surrounds of plantlet.

vii. Dried leaves or grasses is placed around the base of the plantlet in order to maintain humidity.

viii. The plantlet is watered twice a day i.e. in early morning and late afternoon for 4 months.

9. Conclusion and future works

This reviews discussed the application of tissue culture techniques as an alternative mean of asexual propagation of important plants. The advantages of tissue culture techniques allow the propagation of recalcitrant plants including oil palms, endangered plants species as well as seasonal dependent plants [8]. This technique required small amounts of plant tissues to propagate large scales of plant clones, thus, making it a convenient method for plant breeding. Plantlets regeneration from cultured plant cells and tissues has been achieved in many species of high economic value. Many of studies are aimed to carry out large scale propagation of important trees yielding fuel, timber, pulp, oils and fruits [9–12]. Therefore, tissue culture techniques became an alternative for tree improvement.

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