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Somatic Embryogenesis of Oil Palm (*Elaeis guineensis* Jacq.) from Bud Explants Using Suspension Culture

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Abstract: The propagation of oil palm seeds through tissue culture and somatic embryogenesis is a preferred method due to its ability to produce entire plants without the need for gamete fusion. While somatic embryo proliferation is typically performed on solid media, this approach is less efficient than liquid media, which is both more cost-effective and can lead to better proliferation results. Thus, this study aimed to determine the most effective combination of growth regulators on MS medium through suspension culture propagation. Buds from oil palm plants served as the initial explants, with embryogenic callus utilized for suspension culture. Bud explants were previously planted on solid MS media with a combination of 2,4-D and BAP to initiate the primary callus which was formed from 97th to 132nd days, with D4B1 (80 mg/L 2,4-D; 2.5 mg/L BAP) media containing the most primary callus. The primary callus was subcultured twice over a 60-day period before embryogenic callus induction. The embryogenic calli formed on D3B1, D3B2, D4B0, and D4B1 medium between the 90th and 120th days. The medium, D4B1 again successfully initiated the embryogenic callus growth, hence it was later used for suspension culture. The fresh weight of calli increased during the first and second subcultures and later declined in the third subculture. The culture process was repeated on a solid MS medium to obtain coleoptilar somatic embryos, with most of them being formed between the 88th and 115th days (40.7%). Somatic embryogenesis is an efficient and cost-effective way of propagating oil palm seeds and the use of a specific combination of growth regulators on MS medium through suspension culture propagation can result in the formation of embryogenic calli and coleoptilar somatic embryos.

Keywords: Bud Explants, Oil Palm, Proliferation, Tissue Culture, Somatic Embryogenesis, Suspension Culture

Introduction

Indonesia relies on oil palm (*Elaeis guineensis* Jacq) as a crucial source of income from its export value and as a raw material for the domestic cooking oil industry. Additionally, the country is exploring the use of palm oil as a renewable energy source, particularly in the hydrocarbon energy industry (Ardana *et al*., 2022; Shearman *et al*., 2013; Romyanon *et al*., 2015). This is because palm oil is widely used in the oleochemical sector to produce fatty acids and alcohol, which are essential ingredients for surfactants used in the petroleum industry (Saxena *et al*., 2017).

The rapid expansion of Indonesian oil palm plantations, concentrated in Sumatra and Kalimantan, demonstrates a revolutionary shift towards sustainable practices (Goh and Potter, 2022). These two islands account for 90% of Indonesia's oil palm plantations and 95% of the country's Crude Palm Oil (CPO) production (Xin *et al*., 2021). To support the expansion and revitalization of the oil palm industry, significant amounts of high-quality seeds are required, along with plant re-actualization and rehabilitation efforts (Soliman *et al*., 2016). However, despite the increasing demand for seeds each year, there is a shortage of available seeds to meet this demand.

The most effective method of propagating superior oil palm seeds is through generative methods, which involve crossing carefully selected parents (Dura × pisifera) to produce Tenera hybrid seeds (Murugesan *et al*., 2008). Vegetative propagation is challenging because oil palm only has one growing point or single apical meristem.

While generative propagation using seeds is effective, it results in a wide range of traits and can be time-consuming (Green *et al*., 2013; Soh *et al*., 2017). Tissue culture is another method used for propagating oil palm plants, but it is only used for 2% of planting materials due to its limited application, despite some advantages over traditional seed propagation (Weckx *et al*., 2019).

Compared to seed propagation, tissue culture offers several advantages, including a faster and more efficient multiplication rate of plant material, the ability to improve existing plant material by creating new plants with superior characteristics, and the potential to produce new plant material through genetic engineering, disease resistant plant selection, and germplasm conservation (Bridgen *et al*., 2018; Oseni *et al*., 2018). However, traditional tissue culture techniques such as a node or meristem cultures are not effective for oil palm micropropagation. Instead, a piece of plant tissue containing a pre-existing meristem, such as an axillary bud or shoot apical meristem, is extracted and cultivated on a culture medium to generate a new shoot (Weckx *et al*., 2019). Somatic embryogenesis, which is the process of differentiating somatic cells into embryonic cells that have totipotent qualities and can form somatic embryos to create entire plants without gamete fusion, has been documented as a successful method for oil palm regeneration (Guan *et al*., 2016).

In this study, liquid media was utilized as a proliferation medium for the formation of embryogenic callus. The use of liquid media has been found to enhance the sensitivity of many species in tissue culture by promoting increased contact between the media and the explants, thereby making them more receptive to growth regulators. The growth regulators, such as Dichlorophenoxyacetic acid (2,4-D) and 6-Benzylamine Purine (BAP), were used in the process of somatic embryo proliferation and early callus development. These substances are synthetic auxins that are commonly used in tissue culture. Additionally, 2,4-D can be used alone or in combination with cytokinins to initiate callus formation. Therefore, the objective of this study was to identify the optimal combination and concentration of 2,4-D and BAP growth regulators in the initiation of embryogenic callus and proliferation of somatic embryos from oil palm bud explants using callus initiation media on MS solid medium and proliferation medium.

Materials and Methods

Preparation of Source Explants

A 1.5-year-old individual oil palm (*Elaeis guineensis* Jacq) tree was planted in PT. Socfindo Bangun Bandar, North Sumatra, was used as a source of bud explants. The plants were removed from mature leaves, the roots were cut and the outer sheath layer was peeled off to expose the layers of buds (3 cm in diameter). The explants were sterilized under the following treatments: Washed with running water, soaked in a detergent solution, rinsed with sterile distilled water thrice, soaked in 15% NaOCl for 10 min, and rinsed with sterile distilled water. The explants were then immersed in 10% NaOCl, rinsed with sterile distilled water, immersed in 96% EtOH for 15 min, rinsed again with sterile distilled water, followed by immersion in a solution of 0.1 g/L $HgCl_2$, and rinsed using sterile distilled water thrice.

Initiation of Primary Calli

Murashige and Skoog (MS) medium was used as a callus initiation medium supplemented with 2,4-D and BAP. Fragments of explant were aseptically inoculated into solid MS medium with the following formulations as presented in Table 1. Additional supplements were added into the MS medium i.e. 100 mg/L of myo-inositol, 0.9 mg/L of nicotinic acid, 5 mg/L of thiamine, 5 mg/L of pyridoxine-HCl, 0.25 mg/L of pantothenic acid, 2 mg/L of glycine, 30 g/L of sucrose and 1.5 g/L of activated charcoal. The pH of the medium was adjusted to 6.0 prior to sterilization. The experimental design employed in this study was a Complete Randomized Design (CRD), consisting of 150 experimental bottles with 15 treatments and ten replicates for each treatment. The cultures were incubated at 25±2°C in 16 h photoperiods in the incubation booth.

Table 1: Experimental concentration of 2,4-D and BAP in MS medium

	Plant growth regulators (mg/L)	
Treatments	2,4-D	BAP
D0B0	0	0.0
D0B1	0	2.5
D0B2	0	5.0
D1B0	20	0.0
D1B1	20	2.5
D1B2	20	5.0
D2B0	40	0.0
D2B1	40	2.5
D2B2	40	5.0
D3B0	60	0.0
D3B1	60	2.5
D3B2	60	5.0
D4B0	80	0.0
D4B1	80	2.5
D4B2	80	5.0

The Proliferation of Embryogenic Calli in Suspension Culture

The primary calli were incubated further in solid MS medium (0.5 mg/L of 2,4-D, 0.1 mg/L of BAP) and incubated at 25±2°C in the dark. The embryogenic calli (100 mg) of nodular phase measuring 0.2-1.0 mm were cut into smaller aggregates and incubated in 10 mL of liquid MS (0.5 mg/L of 2,4-D, 0.1 mg/L of BAP) medium at 100 rpm and 25±2°C in the dark. The fresh weight of calli was measured during the subculture phase. The subculturing was carried out once every two weeks by rejuvenating 10-20% (v/v) of the medium. The suspension cultures were monitored until reaching the globular phase of somatic embryogenesis. Germination of somatic embryos was carried out in solid MS medium supplemented with 0.05 mg/L of BAP, 0.1 mg/L of Indole Butyric Acid (IBA), and 0.1 mg/L of 1-Naphthaleneacetic Acid (NAA) with ten replicates each (Zouine and Hadrami, 2007). The cultures were incubated under a 16 h photoperiod with 2000 lux intensity of white light at 27±2°C and 70-80% relative humidity.

Data Analysis

The percentage of calli induction of each experiment was calculated using the following formula: [(Number of calli/total number of explants)×100%]. Data collected were analyzed using an Analysis of Variance (ANOVA) and further multiple comparison tests using Duncan's Multiple Range Test (DMRT) at a 5% level if significant ($p \leq 0.05$).

Results and Discussion

Oil palm propagation by indirect somatic embryogenesis can be accomplished via bud explants and callus development. The benefits of indirect somatic embryogenesis are rapid, uniform, and enormous scales of embryogenic production. The type and concentration of auxin and cytokinin, as well as their combination, differ between species when it relates to providing the optimum medium (Méndez-Hernández *et al*., 2019).

In this study, the emergence of a primary callus can be detected between the 97th and 132nd days after culture on solid MS medium, which was accompanied by swelling of the explants and the appearance of callus, particularly surrounding the wound. This suggests that a certain period of time is required for the explants to adapt to the new cultural conditions and initiate the callus formation process. The swelling of the explants observed during callus formation is likely due to the accumulation of cells at the site of injury or explant, which subsequently proliferate to form the callus (Ikeuchi *et al*., 2013). Primary callus formation was not observed in all treatments; callus induction only occurred in a small proportion of treatments Table 2.

The results showed that the optimum concentration of growth regulators was between 60 and 80 mg/L for 2,4-D and between 0 and 2.5 mg/L for BAP. The bud plantlets from oil palm were considered successful in producing ideal primary callus. The resulting callus is compact, dense, and bumpy, with a yellowish-white appearance. These characteristics are desirable for callus formation in oil palm tissue culture, as they indicate a healthy and robust callus that is suitable for further propagation and regeneration (Binte Mostafiz and Wagiran, 2018). To initiate the formation of plant calli, auxin is necessary. During the induction stage, explants are placed in a medium that contains auxin and kept in the dark, which triggers the formation of the primary callus. There are different types and amounts of auxin that can be used to induce primary callus formation in various explants.

Typically, when a medium has a high concentration of auxin, activated charcoal is also added to help reduce explant browning, which is a common problem in oil palm tissue culture. The activated charcoal may act as a non-selective adsorbent capable of absorbing up to 99% of growth regulators in the media (Nakasha *et al*., 2016).

Among chemical substances, 2,4-D is the most effective at inducing embryogenic callus in palm species (Pádua *et al*., 2013). This chemical compound can improve the levels of natural auxin and accelerate cell growth, leading to the development of embryogenic callus in large quantities. Embryogenic callus can further give rise to pre-embryoids, which are small white spots that are visible on the surface of leaf explants. Cytokinins such as BAP are known to influence the development of calli by reducing cell wall lignification, thereby promoting the initiation and growth of callus *in vitro* (Arimarsetiowati *et al*., 2022). Somatic embryo induction and regeneration is a highly sensitive process to culture conditions. The formation of callus was rapidly initiated during the 99th day after planting in D4B1 treatment. The development of somatic embryogenesis can be influenced by age, explant type, genotype, interactions between endogenous and exogenous hormones, and nutritional condition (Singh *et al*., 2016; Raji *et al*., 2018).

The formation of the primary callus was achieved between the 94th and 118th days. The calli formed are embryogenic and non-embryogenic. A non-embryogenic callus is yellow-brown in color, granular in structure, and translucent, whereas an embryogenic callus that appears is circular in shape like polarized, yellowish white in color and crumbs in texture, indicating that the callus is in the form of a collection of cells that are easily detached. The optimum concentration to initiate embryogenic callus was obtained in D4B1 (80 mg/L of 2,4-D, 0.25 mg/L of BAP) treatment although not significant under the availability of BAP in MS medium (Table 1).

Table 2: Formation and emergence days of primary and embryogenic callus from bud plantlets of oil palm in MS medium

	Primary callus induction (mean ± S.D) in days			Embryogenic callus induction (mean ± S.D) in days		
Treatments	B0	B1	B2	B0	B1	B2
D0	–	–	–	–	–	–
D1	–	–	–	–	–	–
D2	–	–	–	–	–	–
D3	129±4.7^{e}	122±3.6^{d}	117±2.7^{c}	–	118±3.3^{c}	105±3.5^{b}
D4	108±1.2^{b}	99±1.0^{a}	–	97±1.3^{a}	94±0.7^{a}	–

D0-D4 = 0, 20, 40, 60, 80 mg/L of 2,4-D. B0-B2 = 0, 2.5, 5.0 mg/L of BAP. (+) = Presence; (–) = absence. Lowercase letters indicate significant differences between treatments ($p \leq 0.05$). Different lowercase letters indicate significant differences at the 5% level tested using DMRT. Mean values are obtained from ten replicates (*N*). (–): No growth was detected

Table 3: Callus morphology in MS medium

Treatments	Coloration	Texture
D3B0	Brownish white	Compact
D3B1	Brownish white	Intermediate
D3B2	Yellowish white	Intermediate
D4B0	Yellowish-white	Crumbs
D4B1	Yellowish-white	Crumbs

However, the rate of embryogenic callus production in MS media was only 12%, which was observed in 18 out of 150 tested bottles, which is a relatively low percentage. A prior investigation also showed that callus formation from bud plantlets was the least effective in comparison to other sources, including cotyledonary node, epicotyl, hypocotyl, immature embryo, and leaf in groundnut (Venkatachalam *et al*., 1996). The use of auxin and 2,4-D, dicamba, and picloram in combination resulted in an induction rate of 23.5-23.7% of embryogenic calli in oil palm zygotic embryos (Thuzar *et al*., 2011).

Another study reported that the efficiency of callogenesis or callus production in oil palm leaf explants is just 15% of that of grown explants. Less than half of the callus generated, or around 5%, is capable of differentiation, is embryogenic, and regenerates into new plants in sufficient quantities (Soh *et al*., 2011). The variation in the explant sources and *in vitro* culture techniques used could account for the difference in embryogenic calli production rates.

The callus that formed had a yellowish-white color and was surrounded by a dense nodular structure. It began to develop from the explants as a response to the supplied growth regulator and interactions with the growth medium and environment. The initial formation of the callus involved the swelling of the wound explants, followed by thickening. Additionally, the embryogenic callus had a compact structure, smooth surface, and a yellowish-white color. These observations were made based on the visual appearance of the callus (Bano *et al*., 2022). The callus morphology is presented in Table 3 and Fig. 1.

The embryogenic calli that are formed on the solid medium are typically in the form of clusters, whereas the callus that forms during proliferation in suspension cultures is in the form of crumbs. The formation of white calli is indicative of proper biomass condition and a friable texture. The color changes in the callus can indicate the occurrence of a growth phase and the potential of cells to regenerate (Martínez *et al*., 2018). Colors such as green, white, yellow, and brown suggest that the cells can still actively divide, while black-yellow-brown colors indicate that the cells are aging (Konar *et al*., 2019). The characteristic friable texture of callus is influenced by the hormone auxin, which facilitates the quick elongation of cells and modifies the flexibility of cell walls. This process leads to a loosening of the cell walls, allowing water to enter via osmosis, thereby causing the cells to expand. The lack of lignification in the cell walls of friable calli allows for easy separation during tissue culture techniques, which is an advantage attributed to their low adhesion properties (Ribeiro *et al*., 2015).

The highest fresh weight of callus was obtained in D4B0 and D4B1 treatments. Callus growth is influenced by both the origin of the explants and the growth regulators used, with the addition of external auxin leading to an increase in callus mass, including both fresh and dry weight. This increase in biomass can be linked to an upsurge in cell division and tissue differentiation. Previous studies have shown that the use of 2,4-D significantly promotes callus biomass production (Bano *et al*., 2022).

In this study, embryogenic callus proliferation into somatic embryos was performed utilizing the suspension culture method, with the aim of accelerating growing time and enhancing the multiplication of oil palm tissue cultures. The success of the suspension culture was determined by measuring the fresh weight growth of globular phase somatic embryos generated at each subculture step. The results showed that the fresh weight of the callus remained constant until the third subculture and then declined, indicating a possible influence of inadequate liquid media on the stagnation of somatic embryos during globular phase growth (Fig. 2).

There were several factors that influenced the success of the tissue culture, including the composition of the medium and the environmental conditions during incubation. After producing somatic embryos using suspension culture, the embryos were subcultured onto solid MS media to promote germination. Between the 88th

and 115th days after culture, coleoptile formation in somatic embryos was observed after subculturing in the same medium every two months. The coleoptile displayed growth polarity and germinated to generate shoot primordia. Morphologically, a greenish color developed at the tips of the shoots in somatic embryos (Fig. 3).

The percentage of coleoptile development was around 88.8%, indicating that somatic embryos have the potential to multiply and develop into sprouts. The process of embryogenesis is a sequential development process that begins with promoting somatic embryo maturation and progresses to the regeneration of shoots and roots, ultimately resulting in the formation of appropriate new plantlets. The production of oil palm tissue culture takes at least one and a half years, with the somatic embryo development phase being the most time-consuming, beginning with callogenesis, which takes two to four months, and progressing to embryogenesis, which takes another seven to eight months (Pattarapimol *et al*., 2015).

The mean fresh weight of somatic embryos during the coleoptilar phase was observed to be 0.81 g. The fresh weight of a plant is an indicator of its water content and its growth is reflected by an increase in size and fresh weight, which is attributed to protoplasm increase as cell size expands. Likewise, the average dry weight of callus was found to be 0.168 g, suggesting that the resulting somatic embryos were rich in water content since the dry weight of the somatic embryos produced was more than 70% of the wet weight of somatic embryos (Soomro and Memon, 2007).

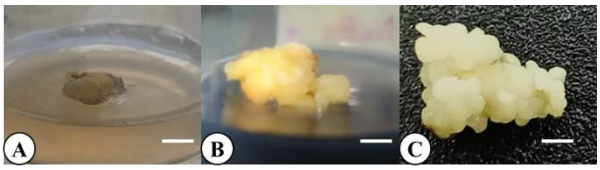

Fig. 1: Appearance of (A) bud plantlet, (B) primary callus, and (C) embryogenic callus of oil palm cultured in MS medium. Scale bar: 5 mm

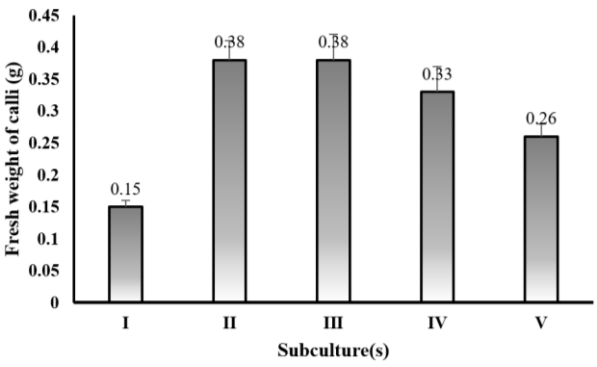

Fig. 2: Profile of fresh weight of embryogenic callus following subculturing. Mean values are obtained from five replicates (N)

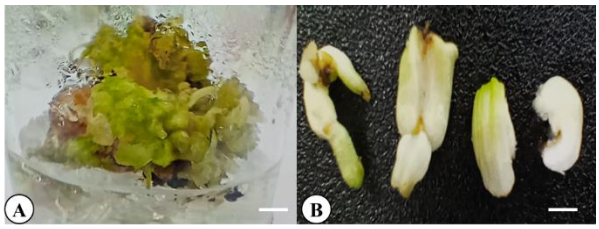

Fig. 3: Appearance of somatic embryos during the coleoptile phase. (A) Clumps of embryos in germination medium. (B) Somatic embryos in various sizes and types. Scale bar: 1 cm

Conclusion

The tissue culture of oil palm (*Elaeis guineensis* Jacq) from bud explants can yield somatic embryos. Utilizing suspension cultures can expedite the growth and multiplication of oil palm tissue cultures. The efficiency of the method is supported by the observed increase in the multiplication rate and the fresh weight gain of the resulting embryogenic callus with each subculture. This suggests that suspension cultures offer a promising approach to improving the efficiency of oil palm tissue culture propagation. Further investigation into the effect of different growth regulator combinations on callus induction and embryogenic callus growth in oil palm bud explants could be explored. Additionally, the use of other culture techniques such as Temporary Immersion System (TIS) or bioreactors could be evaluated to improve the efficiency of somatic embryo proliferation.

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Author's Contributions

Nurul Huda Panggabean: Conception and designed, formal analysis and interpretation of data, funded and drafted the manuscript.

Isnaini Nurwahyuni: Interpretation of data and final approval of the manuscript.

Elimasni: Final approval of manuscript.

Mohammad Basyuni: Formal analysis, experimental and laboratory assistant.

Ethics

This article is original and contains unpublished material. The corresponding author confirms that all of the other authors have read and approved the manuscript and that no ethical issue is involved.

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