

Micropropagation of coconut (*Cocos nucifera* Linn var. *pandan*) through somatic embryogenesis technique

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Article history:

Received: 30 June 2024

Received in revised form: 23

July 2024

Accepted: 3 August 2024

Available Online: 9

December 2024

Keywords:

Coconut,

Cocos nucifera,

In vitro culture,

Plumule,

Somatic

Embryogenesis

DOI:

[https://doi.org/10.26656/fr.2017.8\(S7\).5](https://doi.org/10.26656/fr.2017.8(S7).5)

Abstract

Coconut, also known as *Cocos nucifera*, is one of the most recalcitrant species for *in vitro* regeneration. Conventional coconut breeding has a long lifetime and high heterozygosity, making it a time-consuming, intricate, and costly technique for plant growth. As a result, it is critical to replant most of the world's agricultural land and to develop new soil. This massive undertaking will necessitate planting at least a billion coconut palm trees, which cannot be propagated by seed. Somatic embryogenesis, as a biotechnological alternative, is thus required. Work on this subject has been done in laboratories in numerous countries, with different techniques and explant shapes being investigated. The zygotic embryos are more effective in initiating the callus induction fortified on C1 and C2 formulations added with activated charcoal, gelling agent, and hormone 2, 4- dichlorophenoxyacetic acid (2,4-D). The study has observed that the C1 media successfully initiated callus formation, but the C2 media allowed embryogenic calluses and somatic embryos to develop. From this study, the seedling and regenerated callus were growing well on C3 media. Mature coconut plantlets were acclimated using a combination of sphagnum, sand, and soil mix. The plantlets, on the other hand, could not adapt to their circumstances.

1. Introduction

Pandan coconut, commonly known as the Aromatic Green Dwarf, is the premium coconut of choice due to its sweet coconut water and highly aromatic substance with a strong scent of pandan leaves. It is a high-value plant that generates income for the Malaysian economy's agriculture industry. However, the main problem in the coconut industry is that it is insufficient to reach the coconut market due to the strong demand for pandan hybrids and their limited supply. Coconut is one of the most challenging species to regenerate *in vitro* (Sáenz *et al.*, 2010) (due to a fast-growing need for better or chosen coconut types, particularly those resistant to severe illnesses (Hasnain *et al.*, 2022).

In research on coconut micropropagation, somatic embryogenesis has been established to be a promising strategy for *in vitro* plant regeneration (Chan *et al.*, 1998; Bairu *et al.*, 2011). The plumule explant, which consists of the shoot meristem surrounded by leaf primordia removed from mature zygotic embryos, has proven to be the most sensitive for developing embryogenic callus and somatic embryos (Hornung, 1995; Frank *et al.*, 2000). The utilisation of these explants permitted the development of a reproducible micropropagation

strategy (Frank *et al.*, 2000; Bandupriya *et al.*, 2016), albeit at a low efficiency of four somatic embryos per plumule.

According to Azpeitia *et al.* (2003), brassinosteroids and anti-cytokinins increased the number of somatic embryos per plumule by only 2.5-fold and 1.5-fold, respectively. As a result, for mass micropropagation of elite coconut palms, better regeneration efficiency by somatic embryogenesis is necessary. However, it is essential to note that cloning individuals with known agronomic features would be impossible if plumules were extracted from zygotic embryos produced through cross-pollination.

The zygotic embryo plumule was the most responsive tissue (Chan *et al.*, 1998; Awad *et al.*, 2011). The plumule-based methodology for coconut micropropagation is defined here. It necessitates using Y3-based media supplemented with activated charcoal, gelling agent, sucrose, and growth regulators (Chan *et al.*, 1998; Bunt, 2012). These media allow for the generation of embryogenic callus and somatic embryos, as well as shoot growth and plantlet development (Groll *et al.*, 2002). As a result, this study aims to establish coconut culture stages 1, 2, and 3 using tissue culture

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techniques. Plant tissue culture involves cultivating plants outside the environment and requirements of natural growth by optimising several growth factors for physical, chemical, and environmental factors (Eeuwens, 1976).

The callus culture may improve cultivars and accelerate superior genotype reproduction (Đurkovič and Mišalová, 2008). Callus can be produced by a single differentiated cell, and most callus cells are totipotent and capable of regenerating into complete plantlets (Chan *et al.*, 1998). The calluses came in various shapes and were divided into subgroups based on their macroscopic properties. Depending on the organs they develop, calli, for instance, exhibit varying degrees of organ regeneration; these are also known as shoot, root, or embryonic calli. The cultivation of the coconut palm (*Cocos nucifera* L.) in tropical areas, which gives Kleinholders money and a means of subsistence, is a key one (Azpetitia *et al.*, 2003). The majority of coconut groves around the world require replanting due to senescence or failure due to diseases like *cadang-cadang* in Asia, lethal yellowing in America, lethal illnesses in Africa, and globally distributed *Phytophthora* (Banduptiya *et al.*, 2016).

Plant tissue culture can also grow isolated plant cells, tissues, and organs under axenic conditions *in vitro* to regenerate and propagate entire plants. It is commonly used to describe all types of plant cultures, namely callus, protoplast, cell, anther, meristem, embryo, and organ cultures (Frank *et al.*, 2000). Somatic embryogenesis is a promising *in vitro* approach for the clonal propagation of elite coconut germplasm due to its capacity for large-scale clonal propagation (Muhammed *et al.*, 2016). It was also shown that the cell number in coconut palms was three times higher when cultured in a pH 7.0 medium after 48 hours. Next, adding 5.0 mM glutathione produced a 2-fold increase in cell number, and spermidine at a concentration of 1μM promoted maximum cell growth (Muhammed *et al.*, 2016).

A report by Bairu *et al.* (2011) stated that plant tissue culture involves cultivating plants outside the natural growth environment and requirements by optimising various physical, chemical, and environmental factors for growth (Nguyen *et al.*, 2015). However, the application of this alternative technique is still restrained by some developmental and physiological problems, although improved and meticulous efforts have been made in the plant tissue culture technique. Several *in vitro* plant propagation techniques have been developed, including induction of axillary and adventitious shoots, the culture of isolated meristems, and plant regeneration by organ and somatic embryogenesis (Wilms *et al.*, 2021).

As Zuraida *et al.* (2017) reported, among the diverse types of plant growth regulators evaluated in the coconut palm, optimum callus was observed on MS medium supplemented with 20 mg/L 2,4-D combined with 5 mg/L BAP after 3 - 4 months in culture. Callus induction decreased when 2,4 D was combined with different NAA concentrations (Sáenz *et al.*, 2018). Callus was found to increase with the increase in the concentration of 2,4-D. Furthermore, different coconut varieties and explant types require various concentrations of 2, 4-D for callus induction. Embryogenic callus induction from zygotic embryos required a lower 2,4-D (24 μM) concentration, whereas a high 2,4-D concentration (600 μM) was vital for embryogenic callus induction from plumule and young inflorescence tissues. A combination of a cytokinin, such as kinetic and 2iP (6-(y,y-Dimethylallylamino) purine; both at 10 μM), and 2,4-D (100 μM) has been discovered to increase the production of callus and somatic embryos from microspores in coconut (Kalaipandian *et al.*, 2021).

Several studies have shown that the reduction or removal of 2,4-D from the medium is the only way for coconut somatic embryos to mature (Nguyen *et al.*, 2015). Increasing concentrations of 2,4-D in treating the media composition of 2.5 mg/L to 7.5 mg/L influence the embryogenic callus percentage 56.92 - 91.62% and the percentage of embryogenic callus by administering 2,4-D 10 mg/L. However, not all kopyor coconut explants were cultured to form a callus. Despite being cultured for a long time, explants in the treatment without 2,4-D only showed thickening and did not develop into callus. The explant's cell division and enlargement will be stimulated by adding 2,4-D to the culture medium, which will aid in the formation and growth of callus and improve the natural chemical composition of flavonoids (Zuraida *et al.*, 2017). As a result, due to the low plantlet reproduction of coconut and the increased demand for coconut supply in the coconut sector, alternative propagation by micropagation of coconut pandan was used in this study to identify the optimal culture medium and circumstances for callus induction in coconut *var.* pandan embryos and suckers.

2. Materials and methods

2.1 Plant materials

The cultures were initiated from zygotic embryos and suckers of *Cocos nucifera* Linn. *var.* *pandan*. *Cocos nucifera* Linn. *var.* *pandan* explant samples were collected from Simpang Mengayau, Kudat. An explant used in this study is shown in Figure 1. The samples were stored in a cooler box to maintain freshness before culture.

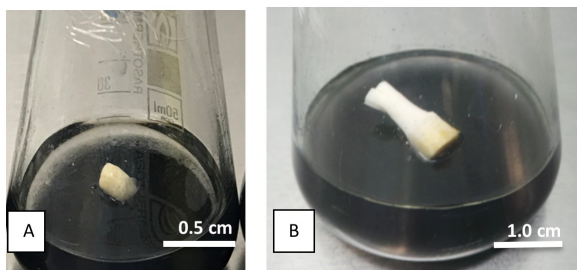


Figure 1. Initiation of coconut *var. pandan* from (A) zygotic embryo of coconut; (B) young suckers aged 3-6 months old. Bar: A and B = 1 cm.

2.2 Media compositions and growth conditions

Basal media C was selected as the essential media to induce callus or embryogenic cells. The basal media used for this experiment were C1, C2, and C3. The C media consists of nutrient macro, micro, iron elements, and vitamins. All the media contained sucrose plus activated charcoal, which were adjusted to pH 5.8 and then solidified with gelrite. The addition of gibberellic acid and 2,4-D in media C1, C2, and C3 was studied. The medium was autoclaved at 121°C for 20 mins. The cultures were maintained in a dark culture room. The room temperature was 25±2°C.

3. Results and discussion

Callus refers to the enormous growth of cells associated with wounding in early plant studies. A report by Gerszberg *et al.* (2016) stated that the callus culture could potentially increase the multiplication rate of the elite genotypes and produce improved cultivars. Callus can be induced from a single differentiated cell, and most callus cells are totipotent and can regenerate into complete plantlets (Davey and Anthony, 2010). The callus had a variety of shapes and was classified into subgroups according to their macroscopic characteristics. For instance, a callus that shows some degree of organ regeneration is known as a shooty, rooty, or embryonic callus, depending on the organs they form.

3.1 Zygotic embryo culture

From the observation, the stage 1 culture was fortified on the C1 medium, and stage 2 media multiplication into the C2 basal medium. From this research, the zygotic embryo's explants positively responded to multiple calluses on media C2. The plantlet was growing well on C3 media. The root media

responded well after optimising the root media on C3 culture media (Figure 2F). After 4 months of culture, approximately 46% of the explants were induced embryos on C1 media (Table 1 and Figure 2B). After 4 months, the callus was increased on C2 media (Figure 2C). The average outcome was 1 flask: 2 flasks. After 12 months, approximately 17% of plantlets were grown on C3 media at 1 flask: 2 plantlets (Figure 2D). The plantlet at Stage 4 was moved to potting media (Figure 2G). However, the plantlet cannot survive after 3 weeks of transplanting. Plant durability in coconut palm causes failure in acclimatisation particularly. As a result, the plantlet of coconut was preferred to grow well in only soil planting media, from cultivated soil to mixed soil and peat moss treatment.

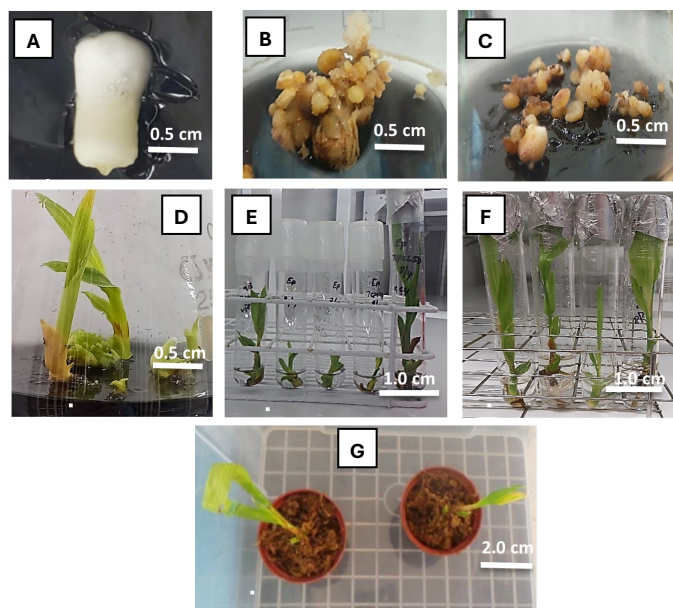


Figure 2. *In vitro* propagation of zygotic embryo coconut *var. pandan*. Bar: A = 0.5 cm, B-D = 0.5 cm; E-F = 1.0 cm; and G = 2.0 cm. Stage 1 media at dark room culture (A- B): where (A) Initiation of zygotic at 1 day of culture and (B) somatic embryogenic cell after 4 months culture. Stage 2 media at lightroom culture (C-E): where (C) Embryogenic or callus cell maturation and multiplication after 6 months culture; (D) plumule emerged after 10 months culture; and (E) Plantlet development after 12 months culture. (F) Root development after 8 months culture (stage 3); and (G) Coconut plantlets after 3 weeks transplanting (Stage 4).

The type and age of the explant also influence the effectiveness of callus initiation and proliferation (Ana *et al.*, 2018). The selection of specific tissues as primary explants depends on the objective of micropropagation, callus production for *in vitro* selection, or a source of

Table 1. Effect of different types of explants on callus induction of coconut after 20 months of initiation.

Type of Explant	Total number of explant produced callus (%)	Shoot regenerated (%)	Total number of plantlet formation	Cumulative contamination (%)	Total direct shoot produced (%)
Zygotic embryo	46	17	10	10	6
suckers	20	5	3	44	16

protoplasts or cells for cultures. The response of an explant to axenic conditions may vary widely between families, genera, species, hybrids, clones, and genotypes, and even within the same genotype grown under different environmental conditions (Awad *et al.*, 2011). Culture conditions such as temperature, light duration, light intensity, and relative humidity are essential factors that affect callus induction of coconut makapuno varieties (Nguyen *et al.*, 2016). Therefore, these factors must be controlled depending on the coconut *in vitro* propagation requirements.

Meanwhile, for the coconut tissue culture, the embryo zygotic is more efficient in initiating the callus or embryogenic cell (Figure 2). All stages 1, 2, and 3 for *in vitro* coconut propagation are shown in Figures 3 and 4. The plantlet cannot survive after 3 weeks of transplanting. Embryo culture micropropagation has several advantages over traditional propagation methods, including the capacity to generate many uniform plantlets in constrained spaces and high-quality, disease-free planting material with efficiency. Facilitating distribution and large-scale planting also increases the quantity of high-quality planting material (Sáenz *et al.*, 2010).

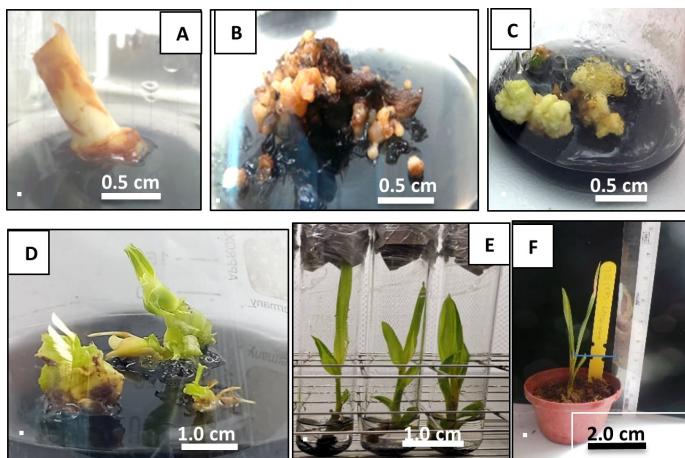


Figure 3. *In vitro* propagation of young suckers' coconut var. pandan. Bar: A-C = 0.5 cm; D-E = 1.0 cm; and F = 2.0 cm. Stage 1 media at dark room culture (A- B): where; (A) Initiation of suckers at 1 day of culture and (B) somatic embryogenic cell formation after 8 months culture. Stage 2 media at lightroom culture (C) Embryogenic or callus cell maturation and multiplication after 8 months culture; (D) plumule emerged after 12 months culture; and (E) Plantlet development after 20 months culture. (F) Coconut plantlet after three weeks of transplanting. Stage 1 media at dark room culture (A- B): where; (A) Initiation of suckers at 1 day of culture and (B) somatic embryogenic cell formation after 8 months culture. Stage 2 media at lightroom culture (C) Embryogenic or callus cell maturation and multiplication after 8 months culture; (D) plumule emerged after 12 months culture; and (E) Plantlet development after 20 months culture. (F) Coconut plantlet after three weeks of transplanting.

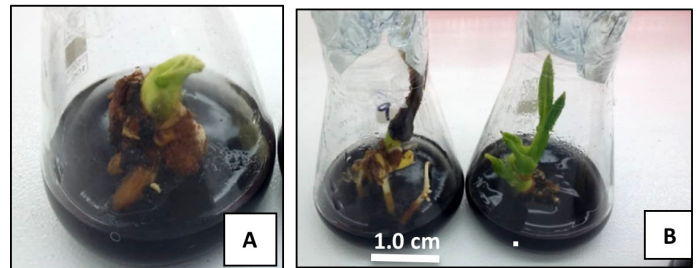


Figure 4. Direct shoot formation from (A) embryo and (B) suckers as an explant. Bar: A and B: 1.0 cm.

Furthermore, a report by Muhammed *et al.* (2016) stated that the zygotic embryo was more responsive regarding forming embryogenic callus, somatic embryos, and their conversion into plantlets. Yields were higher, nearly twofold for calli (60%) and tenfold for calli with somatic embryos (12-24%) than those reported with inflorescence; acclimatisation was successful, and many plantations have been developed in field conditions (Muhammed *et al.*, 2016). Kalaipandian *et al.* (2021) reported that immature zygotic embryos were superior explant tissues for somatic embryogenesis, with a callus induction rate of 50%, compared to just 3% from mature embryos. The middle portion was the best callus formation for embryo sections, ranging from 58% to 83%. The central tissue may cause this problem and contains much of the embryo axis (Kalaipandian *et al.*, 2021).

The observation showed an excellent callogenic response in an embryo culture exposed to the 24-hour dark treatment, with 46% of explants producing calluses. However, the suckers resulted in a poor callogenic response with a 20% number of explant-produced calluses (Table 1). A good callogenic response was found in the C1 medium supplemented with a low concentration of auxins and cytokinin within 4 months of initiation. Direct shoot regeneration from calli derived from the young suckers was successfully initiated by inoculating callus onto a C2 medium fortified with combinations of BAP and NAA added with coconut water also can help to promote cell regeneration, division, and differentiation of meristem cells. About 6% of embryos could regenerate shoots (Figure 4A).

3.2 Suckers culture

From the result, suckers culture gave a 20% number of explant-produced somatic embryogenic cell formation after 8 months of culture in stage 1 media (Table 1 and Figure 3). The explant source from suckers showed the lower number of explants multiplied calluses and regenerated shoot. Meanwhile, the suckers showed embryogenic or callus cell maturation and multiplication after 8 months of culture in stage 2 media in Lightroom, and plumule emerged after 12 months of culture. After 20 months, approximately 3% of plantlets were grown

on C3 media at 1 flask: 1 plantlet. The plantlet at Stage 4 was transferred to potting media (Figure 3F).

Conversely, the plantlets cannot endure after 3 weeks of transplanting. Suckering, synonymous with branching but restricted to the underground portion of the stem (bole), is another rare phenomenon in coconut. Suckering stimulates the opportunity for vegetative propagation of the palms through stimulation of the possible suppressed, rare basal buds in the bole region. The possibility of inducing suckering by causing mechanical injury has been demonstrated in coconut with limited success and is unviable for commercial propagation (Sáenz *et al.*, 2010). Regarding the formation of embryogenic calluses, the production of somatic embryos, and the conversion of somatic embryos into plantlets, zygotic embryonic plumules have proven to be the most responsive tissue (Silvosa-Millado *et al.*, 2020). One embryo can become hundreds of uniform and disease-free seedlings at 270 days after culture (Sandoval-Cancino *et al.*, 2020).

The juvenile tissues have numerous meristematic points, making them more receptive to *in vitro* treatments (Silvosa-Millado *et al.*, 2020). Compared to semi-solid initiation media, a liquid medium allows for better gaseous exchange and optimal nutritional uptake by the germination embryos of coconut (Pérez-Núñez *et al.*, 2006). This problem is because the Y3 medium formulation has higher levels of potassium and iodine and affects the coconut tissue culture environment that is comparable to its native germination habitat on the coastal strand, where the soil is frequently in touch with seawater that is high in potassium and iodine. In addition, it has been discovered that including organic sources of reduced nitrogen is essential for the competitive *in vitro* growth of coconut tissues (Sáenz, *et al.*, 2005).

According to Bandupriya *et al.* (2016), *in vitro* cloning of elite palms via somatic embryogenesis seems a promising alternative for this purpose due to its potential for massive propagation. The advantages to be gained by eliminating the high degree of variation in yield and other selected characteristics that occur during conventional breeding are immense, and therefore, vegetative propagation of coconut utilising tissue culture techniques is highly desirable. The success rate largely depends on the regeneration capacity of this species, which is known to be recalcitrant for *in vitro* culture (Chan *et al.*, 1998). According to Krishnakumar *et al.* (2019), the first experiment indicated that soil texture affects the plantlet growth of the coconut. Also, according to the report by Bunt (2012), peat moss is a partly decomposed plant of the sphagnum genus

harvested from peat bogs that can improve the *in vitro* date palm plantlets acclimatisation rate. Although peat moss is brown, it has a different texture and weight like soil: it is light and spongy and crumbles into tiny splinters. It is hard to wash but retains water like a sponge, minimising surface water drainage once wet. Besides, peat moss can be added to potting soil to lighten it and allow it to retain moisture (Bunt, 2012). According to Āurkoviĉ and Miřalova (2008), from a practical point of view, early tree formation (*Sorbus domestica* L.) during *ex vitro* acclimatisation provides brittle plantlets with mechanical support for better handling of deformations and mechanical injuries during subsequent transplantations. In addition, the soil or potting soil mixture is made from the soil with additional ingredients. Other ingredients may include fertilisers, moisturisers for moisture conservation, or peat moss (Āurkoviĉ and Miřalova (2008).

Coconut somatic embryogenesis contains the induction of embryogenic callus, the formation and development of a somatic embryo, its maturation, then germination, and finally, the recovery of the plantlet formed. Dedifferentiated somatic cells regain their epigenetic and biochemical competence during somatic embryogenesis, forming somatic embryos that develop through a sequence of developmental stages, including zygotic embryogenesis. Somatic embryogenesis in the palm family was first described in oil palm (*Elaeis guineensis* Jacq.) in 1970 and later in coconut in 1977. To date, these techniques have been significantly improved to produce coconut plantlets *in vitro* on a large scale (Kalaipandian *et al.*, 2021). Saenz-Carbonell *et al.* (2016) stated that the coconut research community has identified which explants can trigger morphogenic responses and produce plantlets, all through somatic embryogenesis and learned numerous techniques to optimise this process. From previous studies, the plantlets obtained were true to type and could be successfully acclimatised to *ex vitro* settings while remaining productive in the field. Primary research is being conducted to understand somatic embryogenesis in coconut better. This includes studying the physiology, biochemistry, and molecular mechanisms that produce embryogenic calli and their conversion into plantlets (Saenz-Carbonell *et al.*, (2016).

Another factor affecting *in vitro* germination and micropropagation is the selection of plant growth regulators in media. Plant growth regulators are synthetic substances that have been developed to match naturally occurring organic compounds that are synthesised in higher plants and have an impact on the growth and development of plants. According to Kong *et al.* (2020), two main groups of synthetic hormones that are

particularly important in coconut tissue culture, which is auxins and cytokinin, while other regulators, for example, gibberellins, abscisic acids, and ethylene, are of minor importance.

Auxins are frequently used in micropropagation and are absorbed into nutritional media to promote the growth of calluses, cell suspensions, or organs like meristems, shoot tips, or root tips. They also regulate morphogenesis, primarily in conjunction with cytokinins (Kong *et al.*, 2020). The optimal concentration required depends on the type of plant growth or development needed, the natural levels of auxins within the explant when it is excised, the capability of the cultured tissues to synthesised auxins naturally, and the interaction, if any, between applied synthetics auxins and the natural endogenous substances. The most used auxins are 2, 4-dichlorophenoxyacetic acid (2,4-D), 1-naphthyl acetic acid (NAA), 3-indoleacetic acid (IAA), and 3-indolebutyric acid (IBA) (Nguyen *et al.*, 2015).

Cytokinins such as kinetin (Kn), thidiazuron (TDZ), and BAP (6-benzylaminopurine) can stimulate cell division and induce shoot formation in tissue culture. Besides, cytokinins frequently function as antagonists to auxins. A high ratio of auxin to cytokinin typically promotes the formation of roots, a high ratio of cytokinins to auxins fosters the formation of shoots, and an intermediate ratio encourages the formation of calluses (Kong *et al.*, 2020). It is well known that plant growth regulators utilised in the media significantly impact the success rate of somatic embryogenesis, which produces plantlets. For example, a low concentration of 2,4-D induces the best quantity of embryogenic cells in several Sri Lankan Tall varieties. In contrast, a high concentration of 2,4-D was required for the Malayan Yellow Dwarf to achieve equivalent results. These heterozygous responses exemplify the challenges faced in coconut clonal propagation (Kalaipandian *et al.*, 2021).

Successful plantlet establishment in the field requires a well-established acclimatisation protocol. Any plantlet originating from tissue culture needs to be gradually acclimated to novel environmental conditions. The acclimatisation process for coconut can assume to 3 months. Nonetheless, some coconut plantlets display restricted growth after being transferred to nursery conditions (Kalaipandian *et al.*, 2021). However, in this study, the observation of transplant plantlets at the nursery stage is not discovered due to high mortality in the mist chamber. Therefore, this study has successfully established the tissue culture protocol for propagating Pandan coconut. This newly established protocol could pave the way to overcome the shortage of planting

materials for planted coconut development in Sarawak, assuring the smallholders in coconut industries to meet the global demand for coconut-related products.

4. Conclusion

In conclusion, the micropropagation of coconut extracted from the zygotic embryo and suckers (*Cocos nucifera* var. pandan) has been identified through tissue culture. The most responsive explant to induce callus is sourced from a zygotic embryo with 46% explant-produced callus. However, further study is needed, especially regarding the callus multiplication rate, plantlet formation per explant, and acclimatisation phase.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

We want to express our appreciation to the Sarawak Government through CRAUN Research Sdn. Bhd for financial support.

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