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Callus Induction of Young Leaf Coconut cv. MATAG with Combination of 2,4-Dichlorophenoxyacetic Acid (2,4-D), α-Naphthalene Acetic Acid (NAA) and Benzyl Amino Purin (BAP)

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Abstract

This research was to study in vitro callus induction in Coconut cv MATAG from young leaf explants. Young leaf segments from mature coconut were cultured on Y3 medium supplemented with different concentrations of 2,4-D and a combination of NAA and BAP. Each of these plant growth regulators (PGR) gives different responses toward callus formation, the percentage of explants producing callus, the percentage of callus proliferation, and the morphology of callus. A series of different concentrations were used for 2,4-D (1, 5, 10, 20, 40, 60, 100 mg/L), NAA (1, 3, 5 mg/L) and BAP (1, 3, 5 mg/L) respectively. The range of days of callus formation using 2,4-D treatments is 7 - 12 months, while the 2,4-D combined with NAA is recorded at 2 - 5 months. Despite the variety of different months between these plant growth regulators for callus formation, the percentages of explants producing callus and callus proliferation are different. The highest percentage of explants producing callus (2.9%) was observed at 2,4-D (40 mg/mL), followed by 2.7% at 2,4-D (10.0 mg/mL) with NAA (1 mg/mL). At a concentration of 100 mg/mL of 2,4-D, the highest percentage of callus proliferation was found, as well.

Keywords

Callus Induction, Coconut, NAA, 2,4-D

1. Introduction

The coconut tree, or with the scientific name *Cocos nucifera*, has been gaining attention for the past few years for its own benefits, such as its nutritional and health properties to society. *Cocos nucifera* is believed to have originated in Southeast Asia's coastal areas, including Malaysia, Indonesia, and the Philippines [1]. It is grown in over 90 countries, with the majority of production taking place in Asia and the Pacific. The Philippines, Indonesia, India, Sri Lanka, Thailand, Malaysia, and Papua New Guinea account for over 80% of all coconut plantations globally [2]. The coconut plant has the most applications in the tropics; plant parts are used in the manufacture of food, oil, construction materials, energy, and cosmetics [3].

In vitro culture is a viable alternative for large-scale propagation in the commercial sector. Callus development is one of the in vitro processes. A callus is an amorphous tissue made up of unstructured, dedifferentiated cell masses [4]. A callus can be made from a single differentiated cell, and some of the cells are totipotent, which are able to regenerate as the entire plant body [5]. Induction of cell division, differentiation, and cell development are the three steps of development for a common plant callus [4]. There are a lot of plant growth regulators, but auxins and cytokinins are widely used for callus induction [5]. Auxins are extensively utilised in plant tissue culture for callus induction where these plant growth regulators are involved in cell division, cell elongation, vascular tissue differentiation, rhizogenesis and root formation, embryogenesis, and inhibition of axillary shoot growth [6]. The auxin commonly used for callus induction is 2, 4-D, but NAA and IAA are also used [7]. The aim of this study was to find the best combination of plant growth regulators (2,4-Dichlorophenoxyacetic acid (2,4-D), α-naphthalene acetic acid (NAA), and Benzyl amino purin (BAP) for callus induction and growth of young leaf Coconut cv MATAG.

2. Material and Method

2.1. Sources of Explants and Sterilization

Samples of coconut seedlings were collected from the MARDI Bagan Datok farm in Perak, Malaysia. The sources of explants were the frond of young coconut of about one meter (**Figure 1(a)** and **Figure 1(b)**). These seedlings were subjected to immediate cleaning with detergent and thorough rinsing with tap water The basal portion of the rachis measuring about 30 - 50 cm in length (**Figure 1(c)** and **Figure 1(d)**) was cut, wrapped in a clean plastic bag and transported to the laboratory. Sterilization experiments were performed inside a laminar airflow. The explant was disinfected by spraying with 70% ethanol thrice about a foot

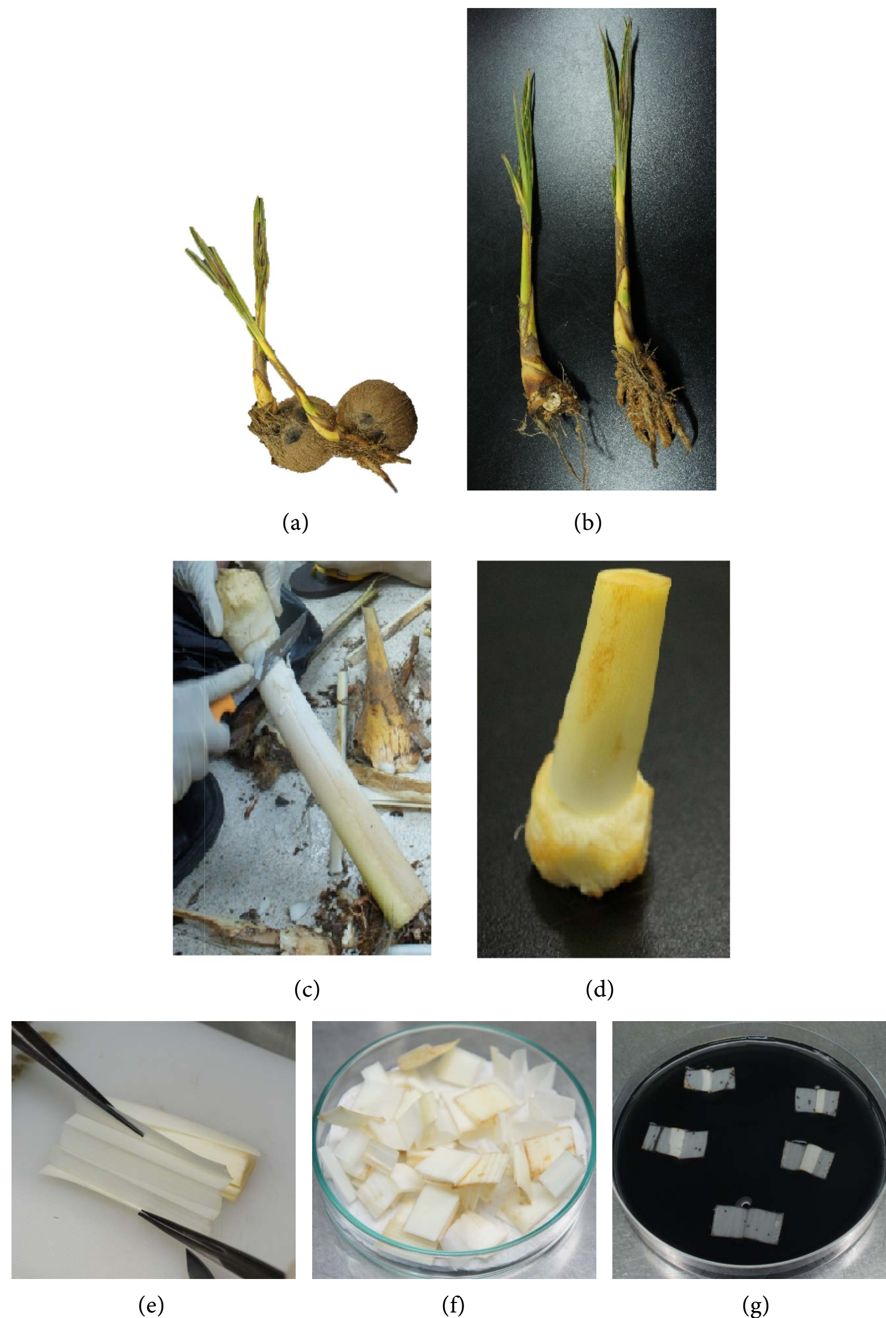

Figure 1. Explants used for callus induction: Young coconut seedling of about one meter ((a), (b)), basal portion of the rachis measuring about 30 - 50 cm in length ((c), (d)), immature leaflets in smaller pieces of 7 - 10 cm, (e) and immature leaf segments of 3 × 1.5 cm in size (f).

away. The preparation of explants involved cutting the white portion of the rachis into smaller pieces at 7 - 10 cm consisting of immature leaflets (**Figure 1(e)**). The explants were surface sterilized by dipping them in 2.0% NaOCl containing several drops of Tween 20 for 15 minutes followed by three times rinse with sterile distilled water. Immature leaf segments of 3 × 1.5 cm in size were used as explants (**Figure 1(f)**) were cultured on the callus induction media.

2.2. Primary Callus Induction

Three experiments were set up to investigate the effects of plant growth regulators on callus induction. The experiments were divided into effects of 2,4-D in combination with NAA or BAP. For callus induction, basal Y3 medium (Eeuwens, 1976) containing 60 g/L sucrose and 0.1% activated charcoal was used. The treatment medium consists of either 2,4-dichlorophenoxyacetic acid (2,4-D) or a combination with naphthaleneacetic acid (NAA) or 6 Benzylaminopurine (BAP) at concentrations of 0, 1.0, 5.0, 10.0, 20.0, 40.0, 60.0, and 100.0 mg/L, respectively. The medium was adjusted to pH 5.8 and then solidified with 3 g/L gelrite before autoclaving at 121˚C for 20 min. The cultures were maintained in a dark culture room at 25 ± 2. The media that could induce primary callus was designated as producing callus medium. The percentage of callus that grew in all treatments was checked every month for the first 15 months of culture and recorded.

2.3. Proliferation of Callus

Clumps of primary callus initiated from the primary callus induction experiment were utilised to induce additional callus or boost callus proliferation. The callus was cultured on the same medium, where it was subcultured 4 - 6 times with an interval of 8 weeks until embryogenic callus formation occurred for 18 months. For 18 months, the callus was cultured on the same medium and subcultured 4 - 6 times with an interval of 8 weeks until embryogenic callus formation occurred at 18 months. The following is the percentage of callus proliferation that was calculated:

$$\text{Proliferation }(\%) = \frac{\text{FW of proliferated callus} - \text{FW of initial callus}}{\text{FW of initial callus}} \times 100$$

3. Results and Discussion

This research was conducted to know the effect of plant growth regulators 2,4-D, NAA and BAP on callus induction, callus proliferation and morphology of callus. **Table 1** shows the effect of 2,4-D on induction of callus from young leaf Coconut cv MATAG. The callus was observed monthly up to 7 months of culture in Y3 medium with different concentrations of 2,4-D plant growth regulator (1, 5, 10, 20, 40, 60, 100 mg/L). All of the calluses that were developed during the early stages of commencement were transparent (**Figure 2(a)** and **Figure 2(b)** and **Figure 2(c)** and **Figure 2(d)**). The culture, environment, type of explants, and hormonal and non-hormonal regulators that may function synergistically in determining the correct induction, proliferation, and regeneration of callus into plantlets all play important role in callus development [8] [9].

The fastest callus formation was observed at 2,4-D (10, 20 mg/L) which took 7 - 9 months with the explant producing callus 0.5% and 0.7% respectively. However, the percentage of callus proliferation (3%) was not good compared to other concentration of 2,4-D. Maximum callus proliferation (20%) was noticed at

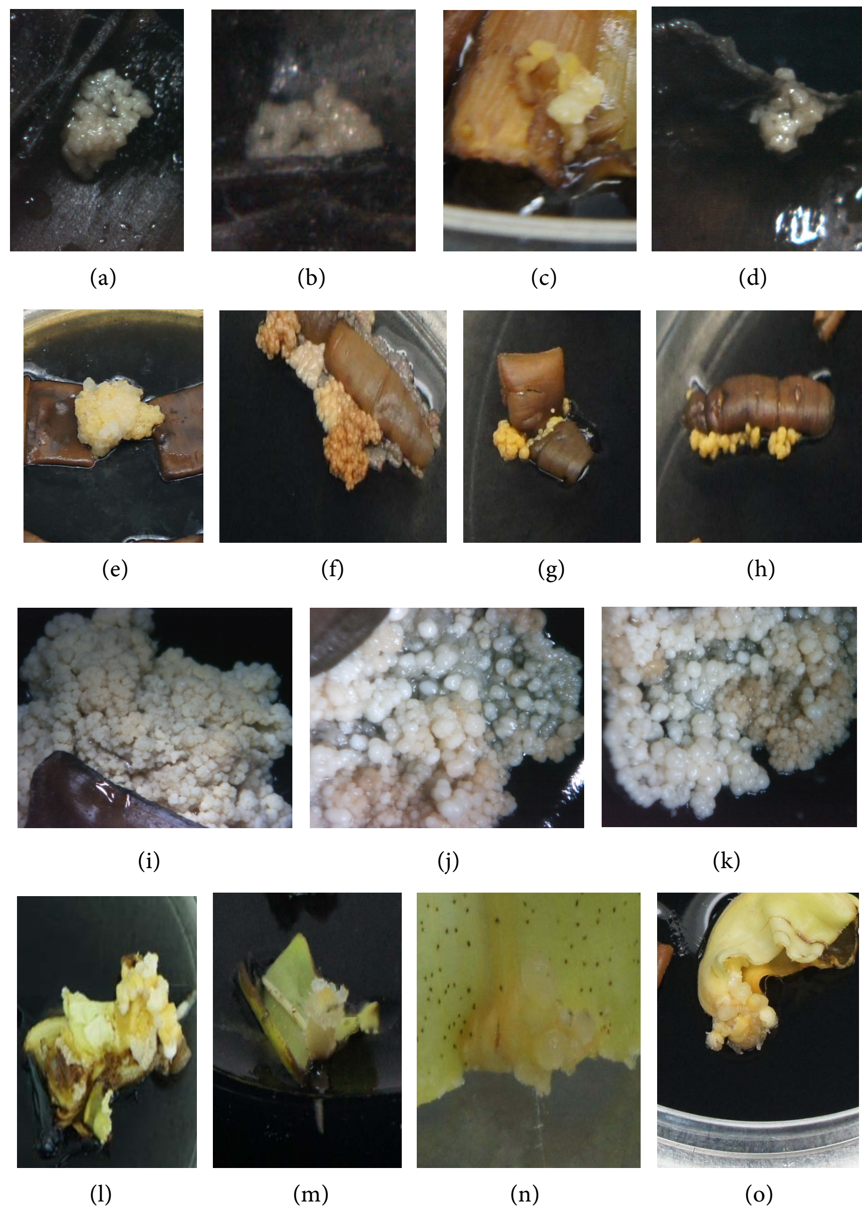

Figure 2. Initiation and callus proliferation of young leaf coconut cv MATAG. Callus obtained after 7 months of culture containing 2,4-D ((a), (b), (c), (d)), friable and yellowish calluses obtained after containing culture on the same medium for another 3 - 5 months containing only 2,4-D ((e), (f), (g), (h)), compact proliferated callus ((i), (j), (k)) and initiated callus obtained after culture on media containing a combination of 2,4-D and NAA ((l), (m), (n), (o)).

2,4-D (100 mg/L) despite the callus formation took around 11 - 12 months with 1.0% of explant producing callus. Friable and yellowish calluses were obtained after continuing culture on the same medium for another 3 - 5 months (Figure 2(e) and Figure 2(f) and Figure 2(g) and Figure 2(h)). There was an increasing pattern shown in the percentage of callus proliferation along with the concentration of 2,4-D. Most of the proliferated callus were compact and white in colour (Figure 2(i) and Figure 2(j) and Figure 2(k)).

Table 1. The effect of 2,4-D on callus induction, proliferation, and morphology in coconut leaves.

Plant growth regulator 2,4-D (mg/L)	Day of callus formation (months)	Explant producing callus (%)	Callus proliferation (%)	Morphology of callus
1	-	0	0	Early stage initiation, all calluses were translucent. After 3 - 5 months, callus turned friable and yellowish.
5	-	0	0	
10	7 - 9	0.5	3	
20	7 - 9	0.7	3	
40	11 - 12	2.9	5	
60	11 - 12	1.5.0	8	
100	11 - 12	1.0	20	

Research from [10] focused on induction and proliferation of callus in *Abies koreana* observed from the results of the range analysis, 2,4-D had the strongest effect on callus proliferation. The greatest proliferation ratio was 1147.6%, measured in the P14 at 2,4-D (3.0 mg·L^{-1}). According to [11] at concentrations of 2 and 4 mg L^{-1} 2,4-D, the percentage of callus induction on leaf explants was 100% until the sixth week of culture and then decreased become 81.3%. [12] found that 2,4-D was the most effective auxin for callus induction on *Centella.* Another point of view was supported in the subsequent experiments on callus proliferation by [10], where 2,4-D supplementation had a more significant influence as plant growth regulator than BAP or NAA.

Furthermore, the influence of Phyto regulators on callus induction was then determined with 2,4-D (1, 5, 10, 20, 40, 60, 100 mg/L) in combination of synthetic auxin NAA (1, 3, 5 mg/L) at different concentrations. From **Table 2**, the fastest callus formation by month was 2 - 3 months can be observed at 2,4-D (10.0, 20.1 mg/L) combine with NAA (1, 3 mg/L) respectively. Despite taking short time for callus formation, the callus proliferation showed a poor result resulting in none of callus proliferation developed. Same goes with the combination of 2,4-D (60.0 mg/L) and NAA (1 mg/mL) that took a longer time (3 - 5 months) for callus formation also showed the same result. All calluses were observed as compact, and yellowish in colour but there were some adventitious roots developed (**Figure 2(l)** and **Figure 2(m)** and **Figure 2(n)** and **Figure 2(o)**). At 2,4-D (10.0 mg/mL) with NAA (1.0 mg/mL) recorded as the highest percentage explant producing callus (2.7%) compared to other concentration combination. A study from [5] observed that auxins such as 2,4-D and NAA are one of the factors to influence callus induction.

After 5 months of culture, the callus turned browning showing that this combination 2,4-D and NAA not giving a good result towards the percentage of callus proliferation on the young leaf Coconut cv MATAG. The cause of no proliferation occurred was due to browning that occurred to the callus after 5 months

Table 2. The effect of 2,4-D and NAA on callus induction, proliferation, and morphology in coconut leaves.

Plant growth regulator (mg/L)		Day of callus formation	Explant producing callus (%)	Callus proliferation (%)	Morphology of callus
2,4-D	NAA				
1.0	1	-	0	0	
	3	-	0	0	
	5	-	0	0	
5.0	1	-	0	0	
	3	-	0	0	
	5	-	0	0	
10.0	1	2 - 3	2.7	0	All calluses that obtained were compact, translucent and yellowish in colour. Some adventitious roots were developed. The callus turned browning after 5 months of culture, and no proliferation occurred
	3	2 - 3	1.5	0	
	5	-	0	0	
20.1	1	2 - 3	0.7	0	
	3	2 - 3	0.4	0	
	5	-	0	0	
40.1	1	2 - 3	0.5	0	
	3	-	0	0	
	5	-	0	0	
60.0	1	3 - 5	0.1		
	3	-	0	0	
	5	-	0	0	
100.0	1	-	0	0	
	3	-	0	0	
	5	-	0	0	

of culture. Browning is also a frequent occurrence in plant tissue culture, restricting normal plant growth and differentiation and, in severe situations, resulting in plant mortality [13] [14]. Moreover, due to substandard concentrations and combinations, callus browning, and necrosis might develop [5] [7].

The treatment of combination concentration 2,4-D and BAP on young leaf Coconut cv MATAG gave different responses. Different concentrations of 2,4-D (1.0, 5.0, 10.0, 20.1, 40.1, 60.0, 100.0 mg/L) in combination of synthetic auxin BAP (1, 3, 5 mg/L) were used. Combination of these two plant growth regulators caused the calluses that obtained compact, yellowish, and brown in colour. The texture of callus was compact due to the effect of cytokinin that play role in transporting nutrients.

Range of callus formation by month for 2,4-D (10.0, 20.1 and 40.1 mg/mL) with BAP (1, 3 mg/mL) was 3 - 6 months according to **Table 3**. Despite having the same range of months of callus formation, the explant producing callus was differ for each concentration. The highest explant producing callus (2.4%) was from concentration 10.0 mg/mL with BAP (1 mg/mL). Even though the percentage of explant producing callus was the highest compared to others, a poor result which none of callus proliferation developed can be observed at this concentration same goes toward other concentration. At 2,4-D (10.0 mg/mL) with BAP (1.0 mg/mL) recorded as the highest percentage explant producing callus (2.4%) compared to other concentration combination. This result can be supported by research from [7] stated that 2,4-D was particularly effective at callus formation, especially when paired with cytokinin (BA).

Table 3. The effect of 2,4-D and BAP on callus induction, proliferation, and morphology in coconut leaves.

Plant growth regulator (mg/L)		Day of callus formation	Explant producing callus (%)	Callus proliferation (%)	Morphology of callus
2,4-D	BAP				
1.0	1	-	0	0	
	3	-	0	0	
	5	-	0	0	
5.0	1	-	0	0	
	3	-	0	0	
	5	-	0	0	
10.0	1	3 - 6	2.4	0	All calluses that obtained were compact, nodular, yellowish, brown in colour. The callus turned browning after 9 months of culture, and no proliferation occurred
	3	3 - 6	0.5	0	
	5	-	0	0	
20.1	1	3 - 6	0.8	0	
	3	3 - 6	0.1	0	
	5	-	0	0	
40.1	1	3 - 6	0.4	0	
	3	3 - 6	0.1	0	
	5	-	0	0	
60.0	1	-	0	0	
	3	-	0	0	
	5	-	0	0	
100.0	1	-	0	0	
	3	-	0	0	
	5	-	0	0	

After 9 months of culture, the callus turned browning showing that this combination 2,4-D and BAP not giving a good result towards the percentage of callus proliferation on the young leaf Coconut cv MATAG. This result was contrary with a study from [15] demonstrated that when 2,4-D and 6-BA were added to the culture medium, the calli of *Pinus koraiensis* were well maintained. This statement was supported by research from [10] said that 2,4-D and BAP have potential for callus maintenance and proliferation.

4. Conclusion

This research examined the callus induction of young leaf Coconut cv MATAG with a combination of, 2,4-D, *α*-naphthalene acetic acid (NAA) and benzyl amino purin (BAP) on callus formation, percentage of callus proliferation and morphology of callus. Each of the plant growth regulators gives different responses. 2,4-D was the best for callus induction also callus proliferation (20%) compared with combination of NAA and BAP. At the early initiation stage, the calluses that were obtained were translucent. After proliferation, they turned compact and white in colour. With the combination of NAA and BAP, browning occurred to the calluses after 5 and 9 months of culture causing no proliferation to develop. Hence, 2,4-D is recommended for the use for callus induction for young leaf Coconut cv MATAG.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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