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Research paper



Effect of Different basal Media on Callus Growth and Morphology of *Barringtonia Racemosa* L Endosperms Explant

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Abstract

Barringtonia racemosa L. has many medicinal properties especially the fruit and leaf parts. The fruits are used to relief pain and inflammation, the leaves were proved to control high blood pressure whereas the roots barks are effective to treat chicken pox. Due to its medicinal importance for human health, it is essential to conserve this plant. A comparative study of different types of media was performed to study its effect on callus growth of endosperm explant from *B. racemosa*. Types of basal media studied including Murashige and Skoog (MS), Lloyd and McCown Woody Plant (WPM) and Gamborg (B5). The endosperm explant of *B. racemosa* were cultured on different basal MS, WPM and B5 media supplemented with 1.0 mg/L 2,4-D and 1.5 mg/L kinetin. The observation of callus growth and morphological characteristics of callus were done on weekly basis within 4 weeks. After 4 weeks of incubation period, the maximum fresh weight (0.300 ± 0.027g) and dry weight (0.025 ± 0.003g) were recorded from the explants cultured on MS medium followed by WPM and B5 media supplemented with 1.0 mg/L 2,4-D and 1.5 mg/L kinetin. The textures of callus produced from MS were nodular in shape and creamy colour. In conclusion, all the media studied had successfully induced the callus growth of *B. racemosa* with the present of 1.0 mg/L 2,4-D and 1.5 mg/L kinetin.

Keywords: *Barringtonia racemosa L, callus, Murashige and Skoog (MS) medium, Lloyd and McCown Wood Plant (WPM) medium, Gamborg (B5) medium.*

1. Introduction

People had been used various materials from nature as beneficial sources to cure their ailment and maintain a good health. One of the most usable sources from nature are plant, and as a result, large amount of modern drugs had been isolated and processed from various types of plant. These types of plant that contributes to medicinal properties is known as medicinal plant. These medicinal plant have the abilities to help in curing certain diseases due to their pharmaceutically significant contents of bioactive compounds. The compounds are isolated from plant's secondary metabolites. It is estimated about 50,000 plant species from Asian medicines are used as traditional medicines [1]. Besides providing advantages in curing diseases, these medicinal plants also contributed in productions of foods, perfumes and flavonoids which making them growing in popularity across the globe.

Barringtonia racemosa (L.) is a type of woody plant that possesses many benefits for maintaining human health such as lowering the blood pressure whereas and to treat the chicken pox [2]. Besides that, this plant also contains high phenolic content with the presence of diterpines, triterpenoids, steroids and saponins thus, can enhance the antioxidant activities [3]. A part from that, this species also had been proven to potentially use as a plant-derived anti-gouty arthritis remedy because of its anti-inflammatory activities [4]. Pharmacological activities of this species including antibacterial, anti-tumor, anti-nociceptive, anti-oxidant, anti-inflammatory, alpha-glucosidase inhibitor, anti-fungal, anti-tuberculosis, anti-arthritic and anti-diarrhoeal towards different parts of *Barringtonia racemosa* species were proven based on the previous study [5].

The induction of callus through plant tissue culture is one of the techniques in plant biotechnology which had been introduced to sustain the distributions and productions of this species. This technique is useful as it enables the whole plant body to be generated through the massive growth of cells which known as callus. Classification of callus could be made based on their microscopic characteristics. For instance, the formation of friable callus group can be used to establish the cell suspension cultures to study the bioactive compound. Besides that, the optimum callus induction can be obtained by supervising certain factors such as the concentration of plant growth regulator, types of basal media and incubation temperature which may influence the induction potential and morphology of the callus. In this study, *Barringtonia racemosa* (L.) was selected for callus induction procedure on different types of basal media (MS, WPM and B5) supplemented with plant growth regulators. The growth and morphological characteristics of the callus were identified in this study.

2. Methodology

2.1 Plant materials

Plant materials used in this study were the endosperms of early mature fruits of *Barringtonia racemosa* (L.) that had been taken from Universiti Putra Malaysia campus. The voucher specimen (SK 3191/17) of the sample was placed in the Herbarium, Department of Biology, Universiti Putra Malaysia (UPM), Serdang, Selangor. The early matured fruits that were selected for this study were in big size (6-8 cm) of fruits with soft texture of flesh and pale yellow seeds.

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2.2 Seeds sterilization

Early matured fruits of *Baringgtonia racemosa* (L.) were washed under the running tap water to remove excess dirt. The fruits were dissected in order to remove that endosperms. After that, the endosperms were soaked in sterile distilled water followed with 70% (v/v) ethanol for 3 minutes in a sterile beaker. Then, the explants were soaked in 5.25% of sodium hypochlorite solutions (Cholorox) combine with two drops of Tween 80 for 30 minutes. After that, the explants were rinsed with sterile distilled water for three times to remove excess traces of bleach residue.

2.3 Media preparation

Murashige and Skoog's (MS), Lloyd and McCown Woody Plant medium (WPM), Gamborg B5 media were supplemented with 1.0 mg/L 2,4-dichlorophenoxiacetic acid (2,4-D) and 1.5 mg/L kinetin (**Table 1**) as a plant regulator to initiate callus induction. The concentrations of plant growth regulators (PGRs) that used in this study were based on a study by Osman *et al.*, [6]. The basic media were supplemented with 30 g/L of sucrose as energy sources and 3 g/L of agar as the gelling agent. The pH of the media was adjusted to 5.6-5.8 with 1M sodium hydroxide (NaOH) and 1M hydrochloric acid (HCl). The media were autoclaved at 121°C at 100kPa (15 psi) above atmospheric pressure in 1 hour 30 minutes and were cooled for the usage. All the cultures were incubated in incubation room at 25 ± 2°C in the dark condition.

Table 1: Murashige and Skoog's (MS), Lloyd and McCown's woody plant (WPM) and Gamborg B5 media containing 30 g/L of sucrose,3g/L of agar, 1.0 mg/L 2,4-dichlorophenoxiacetic acid (2,4-D) and 1.5 mg/L kinetin.

Nutrient Medium	2,4-D (mg/L)	Kinetin (mg/L)
Murashige and Skoog without hormone (MS0)	0	0
Murashige and Skoog (MS)	1.0	1.5
Lloyd and McCown Woody Plant (WPM)	1.0	1.5
Gamborg (B5)	1.0	1.5

2.4 Explant selection

The explants used for the callus induction were the endosperms of *Barringtonia racemosa* (L.).

2.5 Determination of callus growth of *Barringtonia racemosa* (L.) callus

The endosperms of *Barringtonia racemosa* (L.) were taken as explants for callus induction. The endosperms that had been sterilized were cut into 0.5 cm^2 each. Then, the explants were cultured onto callus induction media supplemented with 1.0 mg/L 2,4-D and 1.5 mg/L kinetin, 30g/L of sucrose and 3g/L of agar in petri dish. The cultured media were incubated at 25 ± 2°C under dark condition. The cultured media were subculture onto fresh media after 4 weeks of incubation. The weekly basis observations were done within 4 weeks. The results of the callus growth for each treatment were recorded based on the fresh weight and dry weight.

2.6 Observation of callus morphological characteristics

The colours and textures of the callus were observed and recorded after 4 weeks of culture.

2.7 Determination of fresh weight and dry weight of callus

The callus was taken out from the petri dish and both of their fresh weight and dry weight were recorded at the end of the first 4 weeks. The callus was weighed and the actual fresh weight were recorded. After that, the callus was placed into the oven at a temperature of 50°C for 48 hours as the preparation to record the dry weight. Two days later, the callus was taken out from the oven and the dry weight were recorded. Then, the callus was placed back into the oven with the same temperature. These steps were repeated for each replicates to achieve a constant dry weight.

2.8 Determination of callus formation percentage

The callus formation percentage was calculated based on the number of explants that were successfully induced the formation of callus [6]. Each six petri dish have three explants. The frequencies were recorded in percentage (%) and were determined based on the formula:

$$\text{Callus formation (\%)} = \frac{\text{Number of explant formed callus}}{\text{Total number of explants cultured}} \times 100\%$$

2.9 Statistical analysis

Statistical analysis that used in this study was One-way ANOVA using SPSS Version 17 software. Data were collected and analysed for any significant difference between treatments ($p \leq 0.05$).

3. Results

3.1 Callus induction percentage

The progress of callus induction in *B. racemosa* was observed and recorded after 4 weeks of incubation. Based on this study, the highest callus induction percentage (100%) was found in the explants cultured on both Murashige and Skoog (MS) and Lloyd and McCown Woody Plant (WPM) media followed by Gamborg (B5) medium (85%) supplemented with 1.0 mg/L 2,4-D and 1.5 mg/L kinetin (**Table 2**). Plant growth regulator 2,4-D and kinetin play an important role in callus induction since no callus was formed when the endosperm explants of *B. racemosa* were placed on Murashige and Skoog (MS) , Lloyd and McCown Woody Plant (WPM) and Gamborg (B5) media without these plant growth regulators. Thus, adding of 2,4-D and kinetin to each medium increased percentage of callus induction by 70%, 68% and 65%, respectively after 4 weeks of culture.

3.2 Callus growth

Rapid callus growth were recorded in 2 weeks using Lloyd and McCown Woody Plant (WPM) media hence, showed that this medium could induce callus faster on average period of 4 weeks. On the contrary, as in Murashige and Skoog (MS) and Gamborg (B5) media, callus growth took longer time which is in 3 weeks with an average periods of 4 weeks after incubation of all replicates (**Table 3**). The maximum callus growth with profuse calli (+++++) were observed in endosperm explants cultured in both Murashige and Skoog (MS) and Lloyd and McCown Woody Plant (WPM) media compared to Gamborg (B5) that showed moderate callus growth (++++) (**Table 3**).

3.3 Fresh weight and dry weight

Based on **Fig. 1** and **Fig. 2**, the maximum fresh weight (0.300 ± 0.027g) and dry weight (0.025 ± 0.003g) were recorded from the explants cultured on Murashige and Skoog (MS) followed by Lloyd and McCown Woody Plant (WPM) and Gamborg (B5) media with plant growth regulators. The callus water content was reported to allocate about 90% of fresh weight [7]. Hence, by referring to the fresh weight readings, the callus generated on Murashige and Skoog (MS) medium contain the high amount of water compared to the others. Besides that, the high fresh and dry weight also can indicate the growth and sizes of the callus formed. Increased in fresh weight showed greater callus sizes. Based on

the one way ANOVA analysis, the callus induction percentage of the endosperm explants from *B. racemosa* were significantly affected by the different types of media used ($p \leq 0.005$).

3.4 Callus colours and textures

The forming of callus from all media showed similar morphological characteristics which they produced creamy and brown yellow colours (**Table 4 and Fig. 3**). Besides that, the textures of callus produced from Murashige and Skoog (MS) and Lloyd and McCown Woody Plant (WPM) media were nodular while on Gamborg (B5) media, the callus produced were completely compact.

Table 2: Callus induction percentage of *B. racemosa* from endosperm explants cultured on three different types of culture media treated with 1.0 mg/L 2,4-D and 1.5 mg/L kinetin after 4 weeks of culture under dark condition.

Week	Callus Induction Percentage (%) in Different Types of Media		
	MS	WPM	B5
1	30	33	20
2	50	56	45
3	80	100	65
4	100	100	85

Table 3: Callus growth of *B. racemosa* from endosperm explants cultured on three different types of culture media treated with 1.0 mg/L 2,4-D and 1.5 mg/L kinetin after 4 weeks of culture under dark condition.

Week	Callus Growth in Medium		
	MS	WPM	B5
1	++	++	+
2	+++	++++	++
3	+++++	+++++	+++
4	+++++	+++++	++++

Callus growth: - (no callus growth), + (very few callus growth), ++ (minor callus growth), +++ (slightly callus growth), ++++ (moderate callus growth) and +++++ (profuse)

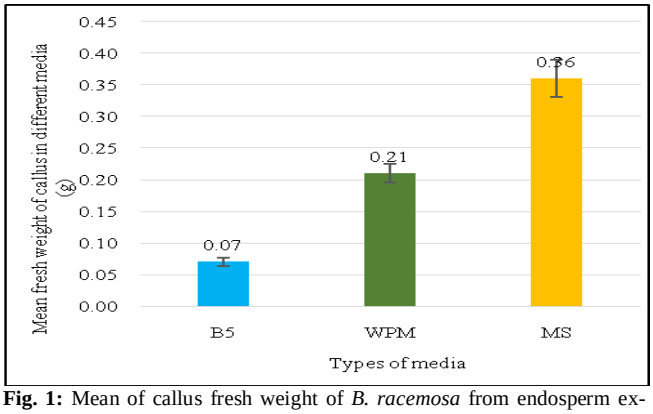


Fig. 1: Mean of callus fresh weight of *B. racemosa* from endosperm explants cultured on three different types of culture media treated with 1.0 mg/L 2,4-D and 1.5 mg/L kinetin after 4 weeks of culture under dark condition.

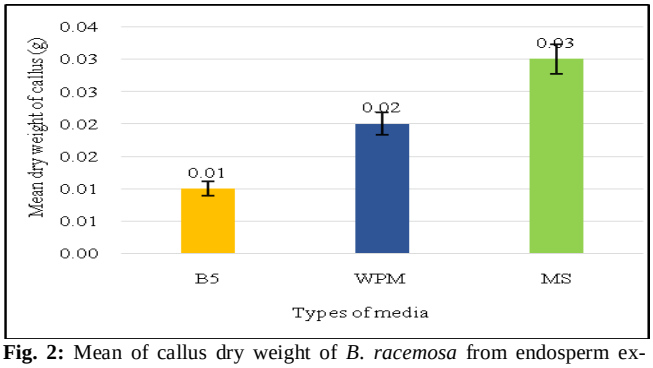
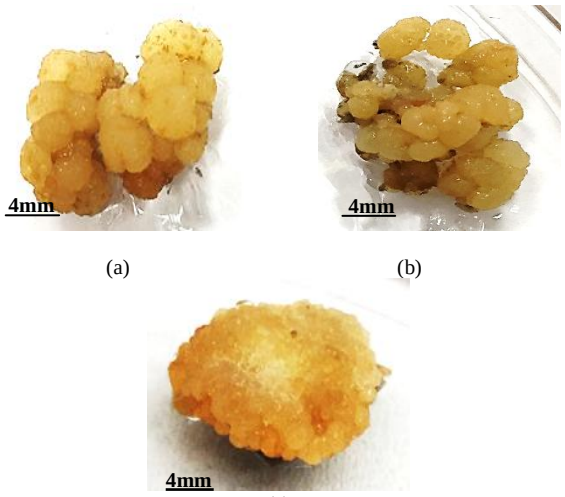


Fig. 2: Mean of callus dry weight of *B. racemosa* from endosperm explants cultured on three different types of culture media treated with 1.0 mg/L 2,4-D and 1.5 mg/L kinetin after 4 weeks of culture under dark condition.

Table 4: Morphological characteristics of callus from endosperm explants of *B. racemosa* cultured on three different types of culture media treated with 1.0 mg/L 2,4-D and 1.5 mg/L kinetin after 4 weeks of culture under dark condition.

Week	Morphological Characteristics of Callus in Medium					
	MS		WPM		B5	
	Textures	Colors	Textures	Colors	Textures	Colors
1	compact	creamy yellow	compact	creamy yellow	compact	creamy yellow
2	compact	creamy yellow	compact	creamy yellow	compact	creamy yellow
3	nodular	creamy yellow	nodular	creamy yellow	compact	brown yellow
4	nodular	creamy yellow	nodular	creamy yellow	compact	brown yellow



(a) (b)

(c)

Fig. 3: Formation of callus from endosperm explants of *B. racemosa* cultured on (a) Murashige and Skoog (MS), (b) Lloyd and McCown Woody Plant (WPM) and (c) Gamborg (B5) media treated with 1.0 mg/L 2,4-D and 1.5 mg/L kinetin after 4 weeks of culture under dark condition.

4. Discussion

4.1 The effect of hormonal treatment on callus induction

In this study, 1.0 mg/L 2,4-D and 1.5 mg/L kinetin were used as a plant growth regulators for each medium involving Murashige and Skoog (MS), Lloyd Wood Plant (WPM) and Gamborg (B5) media. Plant growth regulators are often used in a low concentration to initiate plant growth and development [8]. The most extensively studied class of plant growth regulators in plant tissue culture are auxins and cytokinins [9]. This is because, auxins and cytokinins can help in stimulating the divisions and enlargement of plant cells as well as to govern the dedifferentiation or differentiation mechanisms of explants [10]. Di-chlorophenoxy-acetic acid (2,4-D) is a type of auxins that usually being used in plant tissue culture as it able to effectively induce callus growth in many plant species [11]. The ability of 2,4-D in inducing the callus formation is attributed to its significant functions in stimulating the cell divisions of plant cell. 2,4-D is essentially required in callus formation and had been acknowledged to be the most potent among any other types of auxins [12]. Based on the previous study conducted by Osman *et al.*, [6], showed that, the presence of 2,4-D alone was found to delay the formation of callus at concentrations of 1.0 mg/L - 2.0 mg/L in Murashige and Skoog's medium. This result was also been proved by Amiri *et al.*, [13], where the percentage of callus growth for *Solanum tuberosum* L. had significantly decreased as concentration of 2,4-D increased above 3.0 mg/L. This could be due to the nature of 2,4-D that have the ability to function as herbicide at high concentration thus, inhibit the callus formation and development [14]. Besides that, the addition of kinetin which is the type of cytokinins was required in this study to enhance addi-

tional physiological effect in callus formation and development. This is because, the presence of kinetin can help to stimulate cell divisions and control the morphogenesis of cells [11]. Hence, the combination of 2,4-D and kinetin were used to provide a reproducible and efficient medium for callus induction from endosperm explants of *B. racemosa*. 1.0 mg/L 2,4-D and 1.5 mg/L kinetin was found to be the best hormonal treatments because the results from this study revealed that an increased amount of kinetin concentration associated with 2,4-D at a concentration of 1.0 mg/L was able to produce high percentage of callus induction (**Table 2**). In all treatments in this study, it was showed that the basal medium without the presence of 2,4-D and kinetin did not induce any possible callus growth. However, there was a positive result for basal medium which had been supplemented with 2,4-D and kinetin as it allowed formation and development of callus in endosperm culture.

4.2 Effect of basal media on callus induction

Types of basal medium use can give effects on the potential of callus induction and morphology due to the specific nutrients components. In this study, callus induction of *B. racemosa* from endosperm explants in three different types of basal medium including Murashige and Skoog (MS), Lloyd and McCown Woody Plant (WPM) and Gamborg (B5) media supplemented with 1.0 mg/L 2,4-D and 1.5 mg/L kinetin were examined. Basically, most culture from woody plant species would react on Lloyd and McCown Woody Plant (WPM) media compared to other media as this species has low tolerant on high salts contents. However, based on the results which had been obtained in this study, indicated that, Murashige and Skoog's (MS) medium was the most suitable for callus induction as it produced the highest callus growth from endosperm explants of *B. racemosa* than the other two basal media. These can be seen on the amount of fresh weight and dry weight (**Fig. 1 and Fig. 2**) which had been calculated for each explant. Murashige and Skoog's (MS) medium contains highly concentrated of macronutrients compounds for instance potassium nitrate compared to Lloyd and McCown Woody Plant (WPM) medium as it has low amount of macronutrients, phosphorus and nitrogen [15]. On top of that, according to the previous study by Jansen and Cardon [16] stated that Murashige and Skoog's (MS) medium was the best cultured medium for *B. racemosa*. This is because this species favored slightly saline condition. Thus, it will grow well when cultured in this medium. The lowest callus growth from endosperm explants was resulted in Gamborg (B5) medium. This may be due to the levels of inorganic nutrients which help to increase the growth and development of callus in Gamborg (B5) medium are lower than the other two media used in this study. Hence, Gamborg (B5) medium could not support high percentage of callus induction per explant. Based on the results showed in this study, it was proved that the nutrients components and compositions in each basal medium would affect the degree of callus growth and development.

Besides that, further study had been conducted to examine the morphology of callus formed. Obviously observed in the present study, the morphological characteristics of the callus formed was completely difference in each of the basal medium. The endosperm explants cultured on both Murashige and Skoog (MS) and Lloyd and McCown Woody Plant (WPM) media produced nodular callus while on Gamborg (B5) medium, the callus more likely to appear as compact (**Table 4 and Fig. 3**). The textures of the callus for each of these media were considerably as hard. Furthermore, the colours of the callus for each medium were different along the time and this can be seen where the colors of the callus on each medium appeared as creamy yellow on weeks 1 and 2 while, on weeks 3 and 4, the colors formed was brown yellow (**Table 4**). This might be due to the excretion of phenolic substances as the plant tissues contain large number of phenolic compounds. Apart from that, the callus which had been isolated from the Murashige and Skoog (MS) medium were profuse and this is similar with those cultured in Lloyd and McCown Woody Plant (WPM) medium. However, the callus produced in Gamborg (B5) medium were less profuse compared to the other two media (**Table 3**). Based on the morphological characteristics it can be seen that, the callus may vary depending on the types of cultured medium and the species of explants used [17].

In terms of time consuming for the callus to be induced, obviously Lloyd and McCown Woody Plant (WPM) medium showed faster induction response in which 20 out of 30 explants induced moderate callus growth after 2 weeks of culture (**Table 3**). In contrast to Murashige and Skoog (MS) medium, the explants started to induce moderate callus growth in 3 weeks of culture while Gamborg (B5) medium approximately after 4 weeks of culture (**Table 3**). Previously study had been reported by Behbahani *et al.*, [18] proved that Lloyd and McCown Woody Plant (WPM) able to produce earlier callus induction response where it took 3 weeks for the callus to initiate in leaf explants of *B. racemosa* compared to other medium that required 5 weeks.

5. Conclusion

The best basal medium that produced the highest callus induction percentage was Murashige and Skoog (MS) medium supplemented with 1.0 mg/L 2,4-D and 1.5 mg/L kinetin. The different of callus growth and morphological characteristics of callus can be seen based on the result from each type of basal medium used. It is recommended this callus to be used for cell suspension to study the bioactive compound.

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