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Research paper

Callus Formation on Endosperm from Immature and Mature Fruits of *Barringtonia Racemosa*

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Abstract

Barringtonia racemosa is mangroves type of plant which had been extensively utilized in conventional practices for relieving ailments of pain and inflammation. Many studies have been done on ethnobotanical profiles, pharmacological activities and chemical compounds in *Barringtonia racemosa.* However, there is a limited study on callogenesis of this plant particularly from different maturity stage of fruits. The present study is to identify the callogenesis of *Barringtonia racemosa* from endosperm explants of immature and mature fruits in MS medium supplemented with different concentrations of hormones 2,4-Dichlorophenoxyacetic acid (2,4-D) (0, 0.5, 1.0, 1.5 and 2.0 mg/L) and Kinetin (KIN) (0, 0.5, 1.0, 1.5 and 2.0 mg/L). The optimum hormone combination was found in callus grown on endosperm of immature fruits in MS medium supplemented with 1.5 mg/L 2,4-D and 1.0 mg/L KIN. It was also found that the callus in this treatment grew profusely with highest fresh weight (0.513 ± 0.022 g), 100% callus induction and friable callus texture. The callus fresh weight on endosperm explants was higher in immature fruits compared to mature fruits for all the hormone combinations. Therefore, callogenesis were found more efficient from endosperm explant of immature fruits in *Barringtonia racemosa* species.

Keywords: *Barringtonia racemosa, callogenesis, endosperm, immature fruits, mature fruits*

1. Introduction

Medicinal plants have been used extensively by natives or medical practitioner to treat diseases. *Barringtonia racemosa* Linn. (Family Lecythidaceae) is one of the medicinal plants possess various of bioactivity that engaged to habitat in a wet and watery area such as by the side of fresh swamps, river banks, lakes, shore of backwaters and banks of paddy fields. *Barringtonia racemosa* fruits have been used to treat a cough, asthma as well as diarrhea in Sri Lanka, and treat malaria in Africa [1]. Meanwhile, in Malaysia, the shoots of *Barringtonia racemosa* is widely consumed perceiving to gain health benefits. There is also a practice use a paste of the pounded leaves to reduce the itching skin [2]. Many studies have been done on different parts of *Barringtonia racemosa* towards pharmacological activities such as antibacterial, anti-tumor, anti-nociceptive, antioxidant, anti-inflammatory, an alpha-glucosidase inhibitor, anti-fungal, anti-tuberculosis, anti-arthritic and anti-diarrhoeal [3]. The previous study also supported that *Barringtonia racemosa* is a plant-derived anti-gouty arthritis remedy [4].

On the other hand, the ethnomedicinal uses of the *Barringtonia racemosa* fruits are mitigating the pain and inflammation [5] as well as desirable anti-tumour effect in seed extracts [6]. Locally in Malaysia, the fruits of *Barringtonia racemosa* with bitter taste are eaten fresh as a salad or cooked with turmeric coconut stew. The immature fruits are easier to be eaten fresh due to soft flesh meanwhile mature fruits have hard fibrous flesh unpalatable to be eaten.

The use of *in vitro* culture technique is important in the conservation of plant germplasm and in securing valuable products of medicinal and commercial importance. One of the *in vitro* cultures that suitable for mass production is callus culture. In plant tissue culture, the supplemented nutritional medium with single or multiple hormones is important in directing the callus growth [7]. Cytokinins and auxins have extensively been used to induce callus from plant explants in *in vitro* medium [8]. The suitable hormone concentrations used of cytokinin and auxin as well as source of explant is important in the study of callogenesis or callus formation.

Nevertheless, there are yet no reports on the comparison of callus formation from endosperm explants of immature and mature fruits in *Barringtonia racemosa* species. Hence, determining the optimum callus culture is essential in a mass production of cells that can be used in the further study of somatic embryogenesis and suspension culture for long-term plant conservation and supply.

2. Methodology

2.1. Plant materials

The endosperm explants source was chosen from 2 type fruits maturity. Immature and matured fruits of *Barringtonia racemosa* were collected at Universiti Kebangsaan Malaysia (UKM), Bangi, Selangor. The voucher specimen (SK 3191/17) of the sample was deposited in the Herbarium, Department of Biology, Universiti Putra Malaysia (UPM), Serdang, Selangor. The immature fruit selection was chosen from big size ranged 6-8 cm with soft texture of flesh and pale yellow seeds. The matured fruit selection was categorized by big size ranged 6-8 cm with hard, fibrous texture of flesh and pale yellow seeds. The matured fruits were carefully plucked from the tree before fruits reach over mature (dry fibrous flesh).

2.2. Media preparation

Different concentrations and hormone combinations of plant growth regulators 2,4-Dichlorophenoxyacetic acid (2,4-D) (0.0, 0.5, 1.0, 1.5 and 2.0 mg/L) and Kinetin (KIN) (0.0, 0.5, 1.0, 1.5 and 2.0 mg/L) respectively, were used in the study of callus induction of endosperm of *Barringtonia racemosa.* Type of culture media used was Murashige and Skoog [9] (MS) medium. The MS media were supplemented with 30 g/L sucrose, 6 g/L gelrite agar and the pH was adjusted to be within 5.6 to 5.8 after the addition of hormones. The media were autoclaved at 121°C, 1 atm for 15 minutes.

2.3. Preparation of explants

The explants were surface sterilized as determine by Osman et al. [10]. The seeds were taken out from fruits and sterilized by soaking in distilled water for 5 minutes, 70% (v/v) ethanol for 3 minutes and concentrated sodium hypochlorite (NaCIO) (5.25%) with Tween 80 for 30 minutes which all alternately rinsed thoroughly removing detergent remnants. The sterilized seeds were cut and the large inner area of endosperm was diced into small cubic 0.5 cm^3 using sterilized scalpel under aseptic conditions and cultured onto callus induction media. Twenty-five replications of inoculated explants had been prepared for each treatment and the experiments were repeated thrice. The cultures were incubated at 25 ± 2 ^oC in dark condition and subcultured on fresh MS media after four weeks of incubation. The data for callus induction was recorded on daily basis. After 6 weeks of incubation, the data on morphology and callus fresh weight in each treatment were recorded.

2.4. Onset of callus induction and degree of callus growth

The onset of callus initiation was recorded as a callus started to grow from the cut part on explants. After 6 weeks of incubation, the callus formed on the endosperm explants was graded by degree of growth. The degree of growth was determined by the profusions of callus formed and graded as no callus induce, very few calluses, minor callus, slightly callusing, moderate and profuse (**Fig. 1**).

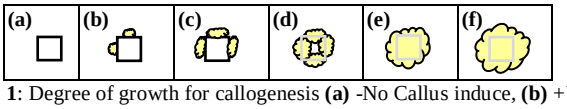

Fig. 1: Degree of growth for callogenesis **(a)** -No Callus induce, **(b)** +Very few calluses, **(c)** ++Minor Callus, **(d)** +++Slightly Callusing, **(e)** ++++Moderate, **(f)** +++++Profuse

2.5. Fresh weight of callus formed and percentage of callus induction

After 6 weeks of incubation, the callus fresh weight and percentage of callus induction were measured. The callus formed were scrapped and isolated from explants. The callus fresh weights (in gram) were measured. The percentage of callus induction was calculated on the basis of the number of explants inoculated and the number of explants formed callus by the following formula:

$$\% \text{ of callus induction} = \frac{\text{No. of explants formed calli}}{\text{No. of explants inoculated}} \times 100\%$$

2.6. Morphological characteristic of callus formed

After 6 weeks of incubation, the callus was characterized based on morphological characteristic includes color and texture of callus formed. The callus was divided into three major morphological categories:
1) watery, pale yellow, and friable, 2) Yellow and nodular with soft texture, 3) Yellow and compact.

2.7. Statistical analysis

Data were analysed using SPSS software version 20. The means of callus growth per treatment were analysed and the significant differences between treatment were measured by ANOVA ($p \leq 0.05$).

3. Results and discussion

Callus could be induced by endosperm explants of both immature and mature fruits using 2,4-D and kinetin. In this study, surface sterilization was done using concentrated sodium hypochlorite, NaCIO (5.25%) and the endosperm was chosen as an explant. It was found that callogenesis was higher from the endosperm explant of immature fruits in all the hormone combinations used.

3.1. Onset of callus induction and degree of callus growth

Table 1 shows the effect of 2,4-D and kinetin combinations towards callus initiation day and degree of growth from endosperm explants of immature and mature fruits. The endosperm explants were found responsive to induce callus at the second week of incubation. Callogenesis started as early as 11 days from both endosperm explants of immature and mature fruits. Hormone treatment combination 1.5 mg/L 2,4-D + 1.0 mg/L KIN for both explants acquired callus formed as early as 11 days with the highest degree of growth (+++++) after 6 weeks of incubation (**Table 1**).

Table 1: Effect of hormone combinations 2,4-D and KIN on the onset of callus induction and degree of callus growth from endosperm explants of mature and immature *Barringtonia racemosa* fruits.

Hormone Concentration (mg/L)		Immature Endosperm		Mature Endosperm	
2,4-D	KIN	Onset of callus induction (day)	Degree of growth after 6 weeks of incubation	Onset of callus induction (day)	Degree of growth after 6 weeks of incubation
0.0	0.0	0	-	0	-
0.5	0.0	14	+++	11	+++
1.0	0.0	15	+++	15	+++
1.5	0.0	15	+++	11	+++
2.0	0.0	15	+++	11	+++
0.5	0.5	12	+++	17	++
1.0	0.5	13	+++	11	+++
1.5	0.5	13	++++	13	+++
2.0	0.5	12	+++	15	+++
0.5	1.0	13	+++	13	+++
1.0	1.0	13	+++	15	+++
1.5	1.0	11	+++++	11	+++++
2.0	1.0	13	+++	15	+++
0.5	1.5	14	+++	15	+++
1.0	1.5	11	++++	11	++
1.5	1.5	13	+++	17	++
2.0	1.5	13	++++	11	++
0.5	2.0	16	+++	13	+++
1.0	2.0	16	+++	17	++
1.5	2.0	15	+++	17	++
2.0	2.0	14	+++	11	++
0.0	0.5	0	-	0	-
0.0	1.0	0	-	0	-
0.0	1.5	0	-	0	-
0.0	2.0	0	-	0	-

*-No Callus induce, +Very few Calluses, ++Minor Callus, +++Slightly Callusing, ++++Moderate, +++++Profuse

In comparison, endosperm explant of mature fruits that were cultured in MS medium with 7 hormone combinations of 0.5 mg/L 2,4-D + 0.0 mg/L KIN, 1.5 mg/L 2,4-D + 0.0 mg/L KIN, 2.0 mg/L 2,4-D + 0.0 mg/L KIN, 1.0 mg/L 2,4-D + 0.5 mg/L KIN, 1.0 mg/L 2,4-D + 1.5 mg/L KIN, 2.0 mg/L 2,4-D + 1.5 mg/L KIN and 2.0 mg/L 2,4-D + 2.0 mg/L KIN were found to form callus earlier than endosperm explant of immature fruits. However, these treatments were not grown profusely after 6 weeks of incubation and degrees of growth on endosperm explant of immature fruits were greater despite the late onset of callus induction. These show that the endosperm explant of mature fruits induced early but callus degree of growth were lesser than in endosperm explant of immature fruits after 6 weeks of incubation.

Callus initiation was triggered in all treatments of MS media supplemented with 2,4-D. It was found that MS medium without hormone and MS media contain only kinetin did not induce any callus growth. Balancing hormone cytokinin and auxin is important to form callus cell. The hormone kinetin under group cytokinin is used to stimulate cell division and control morphogenesis of callus. A 2,4-D is synthetic auxin was added to induce callus due to it can revert cells in the explant to dedifferentiated state and begin to divide [11]. The presence of hormone 2,4-D in this study is essentially important hormone to induce callus from the explants even though the hormone kinetin was absent. The 2,4-D and kinetin hormone combinations have been reported greatly induce callus in plants like *Arabidopsis thaliana* [12], five citrus rootstock species [13], *Nigella sativa* [14] and *Glycine wightii* [15].

3.2. Fresh weight and percentage of callus induction

The effect 2,4-D and kinetin combinations produced a variety of callus fresh weight and high induction percentage from the both endosperm explants of immature and mature fruits after 6 weeks of incubation (**Table 2**). The optimum treatment was determined by considering the highest callus induction percentage and fresh weight.

The maximum callus proliferation after 6 weeks of incubation was notable from callus fresh weights on endosperm explant of immature fruits were higher than callus fresh weight on endosperm explant of mature fruits in all hormone combinations. From the aspect of callus proliferation in all endosperm explant of immature fruits culture with hormone combinations, the high callus fresh weight (**Table 2**) were correspondingly relevance to the high degree of growth as shown in **Table 1**. This indicated that the maturities of fruits influence the proliferation of callus despite the late grow. The combination of 1.5 mg/L 2,4-D + 1.0 mg/L KIN for both endosperm explants of immature and mature fruits produced the highest callus grow in profuse graded (+++++). However, the fresh weight was found higher in endosperm explant of immature fruits (0.513 ± 0.022 g) as compared to endosperm explant of mature fruits (0.371 ± 0.015 g). This treatment was also significantly different ($p \leq 0.05$) as compared to all treatments in the respective endosperm explants from each immature and mature fruits. Thus, the high fresh weight implied that there was a greater production of callus cells from endosperm explant of immature fruits. In this study, the fresh weight and percentage of callus induction on endosperm from the immature fruits were influenced by the big size of fruits ranged 6-8 cm. The big sizes of immature fruits chosen as a sample in the current study associated with cell expansion period within fruit growth pattern. The cell expansion sustained the fruit growth to reach its almost final size before ripening [16]. Previously, a study on fruit size impact towards the callus induction of mango found that higher size of immature fruits results in better callogenesis as compared to the smaller size of immature fruits [17]. Therefore, big size of immature fruits in the current study contributes to the high percentage range of callus induction.

Table 2: Effect of hormone combinations 2,4-D and KIN on callus fresh weight and callus induction percentage from endosperm explant of mature and immature *Barringtonia racemosa* fruits.

Hormone Concentration (mg/L)		Immature Endosperm		Mature Endosperm	
2,4-D	KIN	Fresh weight (g) (mean±SEM)	Callus induction percentage (%)	Fresh weight (g) (mean±SEM)	Callus induction percentage (%)
0.0	0.0	NR	0.0	NR	0.0
0.5	0.0	0.268 ± 0.016^{h}	100	0.248 ± 0.019^{bcd}	91.1
1.0	0.0	0.252 ± 0.013^{h}	100	0.250 ± 0.014^{bcd}	100
1.5	0.0	0.305 ± 0.013^{efgh}	100	0.276 ± 0.019^{bc}	100
2.0	0.0	0.379 ± 0.037^{bcde}	91.1	0.282 ± 0.015^{b}	95.6
0.5	0.5	0.259 ± 0.012^{h}	100	0.199 ± 0.012^{bcde}	95.6
1.0	0.5	0.308 ± 0.017^{efgh}	100	0.211 ± 0.016^{bcde}	91.1
1.5	0.5	0.369 ± 0.008^{cdef}	100	0.271 ± 0.010^{bcd}	100
2.0	0.5	0.326 ± 0.014^{defgh}	100	0.217 ± 0.016^{bcde}	95.6
0.5	1.0	0.293 ± 0.011^{gh}	100	0.265 ± 0.013^{bcd}	95.6
1.0	1.0	0.453 ± 0.014^{ab}	100	0.223 ± 0.009^{bcde}	100
1.5	1.0	0.513 ± 0.022^{a}	100	0.371 ± 0.015^{a}	100
2.0	1.0	0.416 ± 0.015^{bc}	100	0.240 ± 0.017^{bcd}	100
0.5	1.5	0.317 ± 0.011^{efgh}	100	0.210 ± 0.012^{bcde}	88.9
1.0	1.5	0.395 ± 0.013^{bcd}	100	0.190 ± 0.012^{cde}	88.9
1.5	1.5	0.427 ± 0.016^{bc}	100	0.198 ± 0.015^{bcde}	88.9
2.0	1.5	0.505 ± 0.022^{a}	100	0.182 ± 0.008^{de}	100
0.5	2.0	0.270 ± 0.013^{h}	100	0.253 ± 0.010^{bcd}	100
1.0	2.0	0.302 ± 0.017^{fgh}	100	0.153 ± 0.018^{e}	80
1.5	2.0	0.299 ± 0.013^{fgh}	100	0.150 ± 0.008^{e}	95.6
2.0	2.0	0.364 ± 0.018^{cdefg}	95.6	0.200 ± 0.010^{bcde}	95.6
0.0	0.5	NR	0.0	NR	0.0
0.0	1.0	NR	0.0	NR	0.0
0.0	1.5	NR	0.0	NR	0.0
0.0	2.0	NR	0.0	NR	0.0

Mean values with different letters in a column are significantly different at $p \leq 0.05$.No response (NR) indicate there is no present of callus formed.

In terms of percentage of callus induction, the scores were ranging from 88.9% to 100% for endosperm explant of mature fruits which had lower minimum score as compared to endosperm explant of immature fruits with 91.1% to 100% except those cultured on MSO (no hormone) and hormone kinetin alone. Generally, immature fruits endosperm induced more callus (100%) than mature fruits endosperm explants. The high callus induction percentage in this study emphasize that area of endosperm could induce greater as compared to previous finding on callus induction from *Barringtonia racemosa* endosperm. Previously, the study that utilized all part endosperm acquired a percentage of callus induction highest at 56.70% from mature fruits of *Barringtonia racemosa* with the optimum treatment at MS medium supplemented with 1.0 mg/L 2,4-D + 1.5 mg/L KIN [10]. Contradictions of a previous study with our study were differences in the maturity stage of fruits sample chosen, as well as the location of sampling. Big size of fruits also contributes to the callogenesis on *Barringtonia racemosa* endosperm explants in the current study.

In **Table 2**, there was no remarkable percentage of callus induction and callus fresh weight on explants in MS medium without hormone and MS media with kinetin hormone only. The zero percentage of callus induction was equivalently corresponding to callus initiation days and degree of growth in the same treatment as referred to **Table 1**. Therefore, hormone 2,4-D play important role in the present study to revert the cell of explant for callus formation and hormone kinetin is added essentially to stimulate cell division.

3.3. Callus color and morphology

Table 3 shows the effect of 2,4-D and kinetin towards color and texture of callus formed on endosperm explants of immature and mature fruits. MS medium supplemented with 1.5 mg/L 2,4-D + 1.0 mg/L KIN of endosperm explants from both immature and mature fruits yielded large, friable and profuse callus with pale yellow in color. Most friable callus was in pale yellow in color and easily disintegrates (**Fig. 2**) and this texture of callus was suitable to be used in liquid medium as cell suspension culture.

Fig. 2: Formation of callus on endosperm explant from immature fruits cultured on optimum treatment 1.5 mg/L 2,4-D + 1.0 mg/L KIN. The callus formed were pale yellow, friable and covered the entire explant.

Meanwhile, other treatments which were slightly friable classified as nodular with a soft texture, yellow color and some were friable (**Fig. 3**). This morphology was found in 2 combinations treatment of endosperm explant from mature fruits as in treatment of 1.0 mg/L 2,4-D + 0.5 mg/L KIN and 1.0 mg/L 2,4-D + 2.0 mg/L KIN. On the other hand, there were more treatments with similar morphology in endosperm explant from immature fruits at least were grew slightly callusing in the 7 combination treatments of 1.0 mg/L 2,4-D + 0.5 mg/L KIN, 1.5 mg/L 2,4-D + 0.5 mg/L KIN, 0.5 mg/L 2,4-D + 1.0 mg/L KIN, 1.0 mg/L 2,4-D + 1.0 mg/L KIN, 2.0 mg/L 2,4-D + 1.0 mg/L KIN, 1.5 mg/L 2,4-D + 1.5 mg/L KIN and 2.0 mg/L 2,4-D + 1.5 mg/L KIN. These texture of callus formed were mostly 75% nodular with a soft texture and 25% were friable. Even though they were some friable texture, the callus unable to disintegrate easily and lead to a formation of clumps in suspension culture.

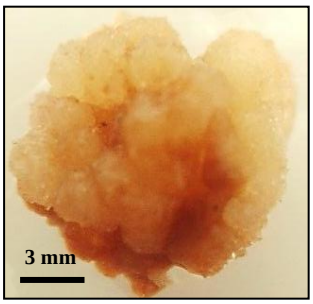

Fig. 3: Nodular with soft texture callus formed on endosperm explant of immature fruits cultured on optimum treatment 1.5 mg/L 2,4-D + 0.5 mg/L KIN.

Other treatments that remarkably compact as well as brittle with yellow color (**Fig. 4**) were not suitable for further studies in suspension culture. Hereby, in the present study, the explant from mature fruits produced more compact callus than explant from immature fruits (**Table 3).** Besides, the previous study of *in vitro* culture on *Jatropha curcas* found that heaviest callus with friable texture was also obtained from the immature seeds [18]. The age of explants has great influenced on the callogenesis [11]. Consequently, different explants age able to affect the viability to induce and proliferates embryogenic callus as well as callus morphogenesis.

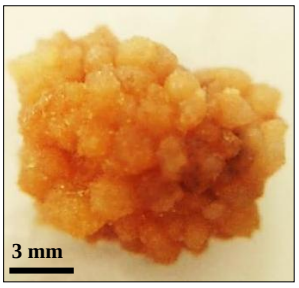

Fig. 4: Compact callus formed on endosperm explant of mature fruits cultured on optimum treatment 0.5 mg/L 2,4-D + 1.0 mg/L KIN.

Table 3: Effect of hormone combinations 2,4-D and KIN towards color and texture of callus formed on the endosperm explants of mature and immature *Barringtonia racemosa* fruits.

Hormone Concentration (mg/L)		Immature Endosperm		Mature Endosperm	
2,4-D	KIN	Color	Texture of callus formed	Color	Texture of callus formed
0.0	0.0	-	No callus induced	-	No callus induced
0.5	0.0	Yellow	Compact	Yellow	Compact
1.0	0.0	Yellow	Compact	Yellow	Compact
1.5	0.0	Yellow	Compact	Yellow	Compact
2.0	0.0	Yellow	Compact	Yellow	Watery and friable
0.5	0.5	Yellow	Compact	Pale Yellow	Compact
1.0	0.5	Pale Yellow	Nodular and some were friable	Pale Yellow	Nodular and some were friable
1.5	0.5	Pale Yellow	Nodular and some were friable	Pale Yellow	Compact
2.0	0.5	Pale Yellow	Watery and friable	Pale Yellow	Watery and friable
0.5	1.0	Yellow	Nodular and some were friable	Yellow	Compact
1.0	1.0	Pale Yellow	Nodular and some were friable	Pale Yellow	Friable
1.5	1.0	Pale Yellow	Large, friable and profuse	Yellow	Large, friable and profuse
2.0	1.0	Yellow	Nodular and some were friable	Yellow	Compact
0.5	1.5	Yellow	Compact	Pale Yellow	Compact
1.0	1.5	Pale Yellow	Watery and friable	Yellow	Compact
1.5	1.5	Yellow	Nodular and some were friable	Yellow	Friable
2.0	1.5	Yellow	Nodular and some were friable	Yellow	Compact
0.5	2.0	Yellow	Compact	Pale Yellow	Watery and friable
1.0	2.0	Yellow	Compact	Yellow	Nodular and some were friable
1.5	2.0	Pale Yellow	Brittle	Yellow	Compact
2.0	2.0	Pale Yellow	Brittle	Pale Yellow	Compact
0.0	0.5	-	No callus induced	-	No callus induced
0.0	1.0	-	No callus induced	-	No callus induced
0.0	1.5	-	No callus induced	-	No callus induced
0.0	2.0	-	No callus induced	-	No callus induced

Even though the callus morphology (**Table 3**) and fresh weight (**Table 2**) varies under influenced of 2,4-D and kinetin combinations, to a certain extent, callus seems to initiate faster in suitable medium whereas in the less appropriate medium the callus induced was slower (**Table 1**). Among all data collected in

this study, endosperm explant from immature fruits cultured on MS medium supplemented with 1.5 mg/L 2,4-D and 1.0 mg/L KIN was found produced the best callogenesis as compare to the all hormone combinations in the same endosperm explant of immature fruits as well as endosperm explant of mature fruits. This treatment produced callus that induced as earliest as 11 days with highest callus formation of 100%, highest callus fresh weight at 0.513 ± 0.022 g, prominent pale yellow color along together friable and profuse callus was formed.

4. Conclusion

Callus formation from endosperm explant of immature fruits was more productive than the callus in endosperm explant of mature fruits. The optimized callogenesis were found more efficient in endosperm explant of immature fruits cultured on MS medium supplemented with 1.5 mg/L 2,4-D and 1.0 mg/L KIN that producing friable callus in large amount. This texture of callus was suitable to be utilized for further study in suspension culture exploration or somatic embryogenesis. This work contributes to the development of an efficient reliable protocol for inducing callus formation whereas hence increasing knowledge about the *in vitro* cultivation of *Barringtonia racemosa.*

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