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Effects of variations in culture media and hormonal treatments upon callus induction potential in endosperm explant of *Barringtonia racemosa* L.

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ABSTRACT

Objective: To induce callus from the medicinally valuable species, *Barringtonia racemosa* L. (*B. racemosa*) whereby the formation of callus is essential for micropropagation studies and *in vitro* plant secondary metabolites production.

Methods: The callus induction potential in *B. racemosa* was assessed from endosperm explant cultured on different culture media and plant hormonal treatments. Lloyd and McCown's woody plant medium and Murashige and Skoog's medium were used in the study as culture media. On the other hand, various concentrations and combinations of 2,4-dichlorophenoxyacetic acid (1.0–2.0 mg/L) and kinetin (0.5–2.5 mg/L) had been incorporated in the culture media to exert the effects of auxin and cytokinin on callus induction.

Results: From the present study, it was found that the profuse [(1.681 ± 0.770) g fresh weight, (0.239 ± 0.239) g dry weight] and friable callus formation was optimally produced with desirable morphology and considerable percentage of callus induction (56.70%) in endosperm explants cultured on 1.0 mg/L 2,4-dichlorophenoxyacetic acid and 1.5 mg/L kinetin in Murashige and Skoog's medium.

Conclusions: A reliable protocol for inducing callus formation of profuse and friable morphology in endosperm explant of *B. racemosa* had therefore been successfully established.

1. Introduction

Barringtonia racemosa L. (*B. racemosa*) is a type of medicinal plant species native to East Africa, Pacific Islands and Southeast Asia including Malaysia. Known as a type of mangrove plants, *B. racemosa* grows well in wet and watery areas such as along fresh water swamps, riverbanks and lakes with the height of approximately 4–8 m but can grow up to 15 m [1–3]. This species gained less attention due to lack of effort in promoting their development and commercialization. Furthermore, in certain regions of the world for instance in Singapore, the species is considered endangered and identified to be at the verge of extinction.

Despite being disregarded especially in modern societies, this species however is a rich source of phytomedicines and has long been used by the elderly as a type of traditional remedies, being included in the dishes whereby the shoots and fruits are usually eaten raw. The medicinal values of the *B. racemosa* have been acknowledged to be among the herbs of choice in various tribes around the world. Furthermore, the pharmacological properties of the species had been scientifically proven and recorded in a number of studies [4]. *B. racemosa* have been documented in Ayurvedic literature in which the fruits of *B. racemosa* are prescribed for the treatment of pain, inflammation and rheumatic conditions [5]. The leaves are traditionally used for treating high blood pressure [3], ulcer, cancer [6] and the pounded leaves, roots and barks are used to reduce itchiness

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and chicken pox [7]. Due to the medicinally valuable properties of the species, the effort to micropropagate this species through plant tissue culture techniques is seen to be useful to ensure its continuous supply.

The induction of callus serves as a basis in plant biotechnology studies in which the development of various plant regeneration studies and somatic embryogenesis may be initiated from callus [8,9]. In addition, the formation of friable callus is essential to initiate and establish a homogeneous cell suspension cultures for plant bioactive compound studies [10]. In order to get the optimum callus induction with the desirable morphogenic response, various callus induction factors may be applied. Variations in external factors such as types of basal culture media, plant growth regulators, sucrose compositions, pH of the culture medium and incubation temperatures are among the factors which may influence the induction potential and morphology of the callus induced [10]. The present study described the works that had been conducted covering the aspect of callus induction potentials under different exogenous factors which were culture media and plant growth regulators supplementation. The effects of those factors had been studied to fulfill the aim of obtaining callus with profuse amount of production and having greatest score of callus weight with friable morphology.

2. Materials and methods

2.1. Surface sterilization of explants

Matured fruits of *B. racemosa* were obtained from Nasuha Herbal Farm, Johor, Malaysia. The fruits were initially washed under running tap water. They were then carefully dissected and the endosperms were taken out from the fruits (Figure 1). Afterwards, surface sterilization procedures for endosperms were carried out by soaking the explants in filtered distilled water followed by 70% (v/v) ethanol for 3 min in a sterile beaker. After that, the explants were soaked in 5.25% sodium hypochlorite solution (Cocorex®) added with two drops of Tween 80 (Sigma, USA) for 30 min. The explants were then thoroughly rinsed with sterile distilled water to remove any traces of remaining detergents.

2.2. Media preparation

Different concentrations and combinations of 2,4-dichlorophenoxyacetic acid (2,4-D) (1.0–2.0 mg/L) and kinetin (0.5–2.5 mg/L) were used in the study. Two types of culture media used were Murashige and Skoog's (MS) medium and Lloyd and McCown's woody plant medium (WPM). The prepared media were consisted of 30 g/L sucrose (R&M Chemicals, UK) and 7 g/L gelrite agar (Duchefa, Netherland) and the pH was adjusted to be within 5.6–5.8 by using 1 mol/L HCl and NaOH after the addition of plant hormones. The media were autoclaved at 121 °C under the pressure of 1.06 kg/cm^2 for 15 min.

2.3. In vitro callus induction

The sterilized endosperm were further aseptically cut into small pieces approximately 0.5 cm^2 and cultured onto callus induction media. Twenty replications of inoculated explants had been prepared for each treatment and the experiments were repeated thrice. The cultures were incubated at (25 ± 2) °C under dark condition and subcultured onto fresh media after four weeks of culture. The observation was done on weekly basis. At the end of six weeks, the formed calli were scraped and isolated from the explants. The data for callus induction were recorded in which the morphology, fresh and dry weights (g) of callus in each treatment were taken.

2.4. Data collection and statistical analysis

The study was conducted according to Completely Randomized Design (CRD). Each week, the percentage of callus induction and callus morphologies from each treatment and media combination were observed. After six weeks of culture period, the percentage of callus induction, weights of calli (fresh weight and dry weight) and the morphology of calli formed were all documented. The percentage of callus induction in each treatment was calculated by the following formula:

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

The optimum treatment was determined by considering the highest callus induction percentage, fresh and dry weight of callus (g) with the optimum intended morphologies of profuse and completely friable calli. The data were presented as mean ± SD. The means comparison and statistical analysis were done using SPSS 17.0 software whereby the effects of hormonal treatments and culture medium types on callus induction were analyzed utilizing paired samples *t*-test at 95% confidence interval. The value of $P < 0.05$ was considered as statistically significant.

3. Results

After six weeks of incubation, the progress of callus induction in *B. racemosa* was observed and recorded. Generally, the calli formed were all yellowish. The highest callus formation with 56.7% callus induction percentage was found in the explants cultured on MS medium supplemented with 1.0 mg/L 2,4-D and 1.5 mg/L kinetin (Table 1). The calli produced from such treatment were found to be completely friable so as to fulfill the desirable morphology (Figure 2) and recorded the greatest fresh [(1.681 ± 0.768) g] and dry weight [(0.239 ± 0.239) g]. After being subcultured (after four weeks of culture period), the calli were proliferated and enlarged (Figure 3). In the present study, most of the calli formed were found to be compact,

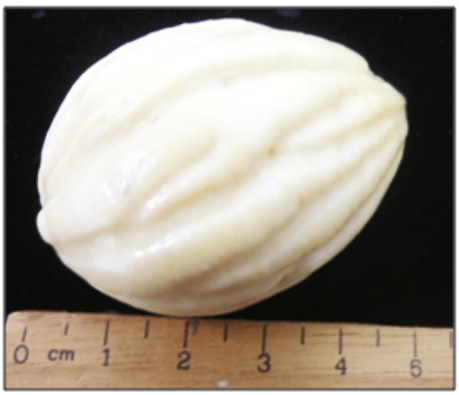

Figure 1. Endosperm of *B. racemosa* which had been taken out from the matured fruit.

Table 1

Callus induction in *B. racemosa* from endosperm explant cultured on MS medium treated with various concentrations and combinations of 2,4-D and kinetin hormones after six weeks of culture at (25 ± 2) °C under dark condition.

Hormonal treatment (mg/L)		Percentage of callus induction	Morphology	Onset of callus induction (week)	Fresh weight (g) (Mean ± SD)	Dry weight (g) (Mean ± SD)
2,4-D	Kinetin					
0.0	0.0	0.0	No callus formation	–	–	–
1.0	0.0	1.7	Compact	2	0.018 ± 0.312	0.003 ± 0.005
1.5	0.0	5.0	Compact	2	0.115 ± 0.199	0.020 ± 0.035
2.0	0.0	28.3	Brittle and nodular	2	0.978 ± 1.039	0.075 ± 0.042
2.5	0.0	50.0	Compact	1	1.667 ± 1.194	0.119 ± 0.039
1.0	0.5	23.3	Compact	1	0.546 ± 0.457	0.051 ± 0.029
1.0	1.0	38.3	Nodular and some were friable	1	0.367 ± 0.154	0.079 ± 0.054
1.0	1.5	56.7	Large, friable, and profuse	1	1.681 ± 0.768	0.239 ± 0.239
1.5	1.0	23.3	Brittle and nodular	2	1.014 ± 1.575	0.070 ± 0.076
1.5	1.5	10.0	Brittle and nodular	1	0.090 ± 0.156	0.021 ± 0.036
1.5	2.0	13.3	Large and some were friable	1	0.257 ± 0.445	0.049 ± 0.085
2.0	1.5	0.0	No callus formation	–	–	–
2.0	2.0	8.3	Friable	1	0.111 ± 0.192	0.034 ± 0.059
2.0	2.5	10.3	Brittle and nodular	1	0.518 ± 0.826	0.042 ± 0.054

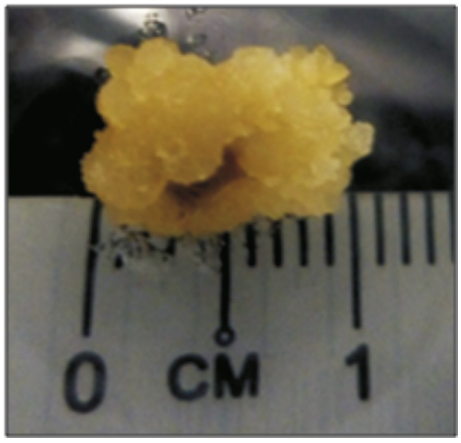

Figure 2. Formation of callus from endosperm explant cultured on optimum treatment [1.0 mg/L 2,4-D and 1.5 mg/L kinetin on MS medium after five weeks of culture at (25 ± 2) °C under dark condition].
The calli formed were yellowish, friable and covered almost the entire explants.

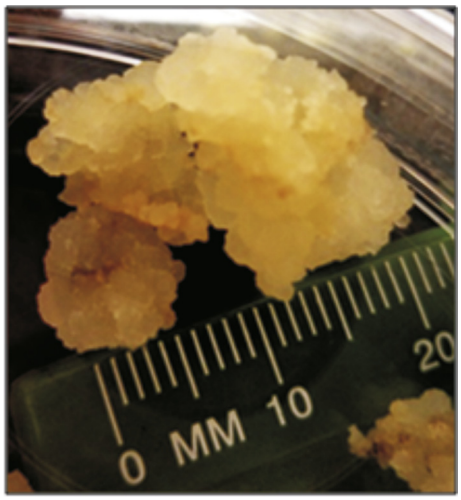

Figure 3. The proliferation and enlargement of friable calli formed from optimum treatment (1.0 mg/L 2,4-D and 1.5 mg/L kinetin in MS medium) after being isolated and subcultured onto fresh medium after six weeks of culture.

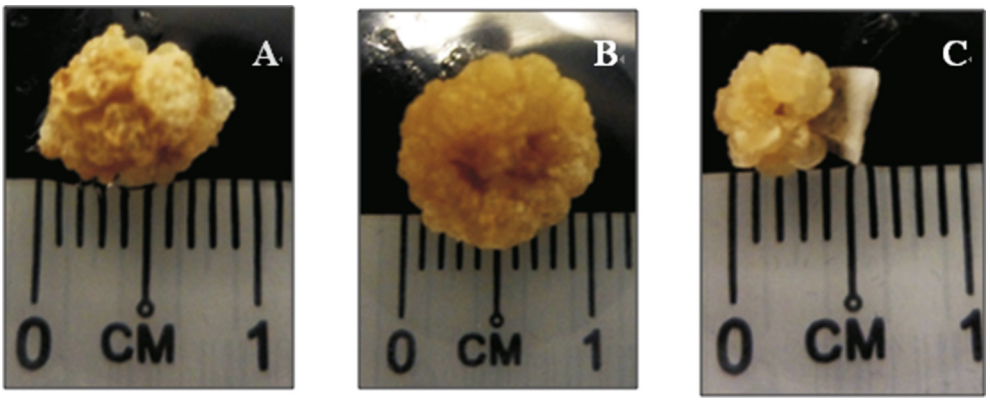

Figure 4. Examples of callus with compact (A and B) and brittle and nodular (C) morphologies.

nodular and brittle (Figure 4). And the findings were prominent in those cultured on WPM medium whereby only one treatment produced friable calli (Table 2). On the contrary, four out of five treatments which produced friable calli in this study were derived from those cultured on MS medium. In terms of percentage of callus induction, the scores were ranging from 1.7% to 56.7% except those cultured on the media treated with 2.0 mg/

Table 2
Callus induction in *B. racemosa* from endosperm explant cultured on WPM medium treated with various concentrations and combinations of 2,4-D and kinetin hormones after six weeks of culture at (25 ± 2) °C under dark condition.

Hormonal treatment (mg/L)		Percentage of callus induction	Morphology	Onset of callus induction (week)	Fresh weight (g) (Mean ± SD)	Dry weight (g) (Mean ± SD)
2,4-D	Kinetin					
0.0	0.0	0.0	No callus formation	–	–	–
1.0	0.0	66.7	Compact	1	0.506 ± 0.139	0.106 ± 0.035
1.5	0.0	33.3	Compact	1	0.357 ± 0.048	0.074 ± 0.033
2.0	0.0	41.7	Compact	1	0.279 ± 0.099	0.144 ± 0.155
2.5	0.0	1.7	Friable	1	0.026 ± 0.045	0.007 ± 0.012
1.0	0.5	26.7	Compact	1	0.170 ± 0.027	0.031 ± 0.001
1.0	1.0	23.3	Compact	2	0.172 ± 0.252	0.041 ± 0.036
1.0	1.5	18.3	Compact	1	0.197 ± 0.171	0.117 ± 0.149
1.5	1.0	18.3	Compact	1	0.294 ± 0.333	0.067 ± 0.060
1.5	1.5	1.7	Brittle and nodular	4	0.002 ± 0.004	0.001 ± 0.002
1.5	2.0	11.7	Brittle and nodular	1	0.198 ± 0.208	0.040 ± 0.029
2.0	1.5	10.0	Brittle and nodular	1	0.096 ± 0.076	0.009 ± 0.011
2.0	2.0	15.0	Brittle and nodular	1	0.140 ± 0.119	0.040 ± 0.022
2.0	2.5	10.0	Compact	1	0.127 ± 0.159	0.020 ± 0.026

L 2,4-D and 1.5 mg/L kinetin on MS medium which produced no callus at all.

4. Discussion

4.1. Effects of hormonal treatments on callus induction

Plant growth regulators are synthetic molecules used in plants and supplemented at a relatively low concentrations to work as signaling compounds for plant growth and development [11]. They can be classified into different types according to their molecular structures and physiological functions. The most extensively used and studied class of plant growth regulators in plant tissue cultures are auxin and cytokinin [8]. 2,4-D which is known as a type of auxin has been acknowledged to effectively induce callus formation in many plant species.

The result from this study revealed that the presence of 2,4-D in the culture media was essentially required to induce callus formation in this species even though the cytokinin was absent. The effectiveness of 2,4-D in inducing the formation of callus is attributed to its main characteristic which can stimulate cell division of plant tissues and strongly suppress organogenesis. It is also considered to be the most potent among any other commonly used auxins [10]. Nevertheless, in the current study, the presence of 2,4-D alone at the concentration of 1.0 mg/L–2.0 mg/L in MS medium was found to delay the formation of calli in which the calli only started to form during the second week of culture. Therefore, the addition of kinetin is required to exert additional physiological effect. The optimum treatment for callus induction in this study was identified in MS medium supplemented with 1.0 mg/L 2,4-D and 1.5 mg/L kinetin. The findings revealed that the supplementation of kinetin at an optimum concentration and combination with 2,4-D is required to produce calli with the desirable morphology. The hormonal combination of 2,4-D and kinetin was previously found to be effective in producing optimum callus induction in *Aquilaria malaccensis* Lam, in which 70%–73% of callus induction was recorded [12].

From the preliminary study conducted previously, the supplementation of singly present kinetin in the culture media did not promote callus induction and was not useful in promoting the formation of profuse calli (data not shown). However, the results from this study had shown that the addition of kinetin in the culture media in combination with 2,4-D was fruitful in producing callus. This is in contrast to Rashid *et al*. in which they found that the addition of kinetin affected the callus formation negatively in *Triticum aestivum* [13]. By looking at the trend of callus formation in this study, an increasing induction percentage was noted associated with an increase in kinetin concentration supplemented together with 2,4-D hormone at the concentration of 1.0 mg/L and 1.5 mg/L. In addition, the positive effect was also noted especially in the media with 1.0 mg/L 2,4-D whereby the morphogenic responses were identified to be improved as the friability was enhanced with an increase in the concentration of kinetin (Table 1). According to the statistical analysis conducted, hormonal combinations do have significant effect towards the formation of callus in this study whereby the value of P is <0.05.

4.2. Effects of culture media on callus induction

The nutrition components in each medium do affect the callus induction potential hence the morphology of calli formed. Obviously observed in the present study, the friability status of the calli formed was completely different in which all the explants cultured on WPM medium produced compact and nodular calli except those cultured on 2.5 mg/L 2,4-D while some treatments used MS medium produced friable calli. Apart from that, it was clearly observed that the calli isolated from WPM culture media were smaller and less profuse than those cultured on MS media (Table 2). Similarly, considering the effects of using WPM medium, a study on plant regeneration in bay leaf tree (*Cinnamomum tamala*) by Sharma and Nautiyal which used WPM as their sole culture medium reported that almost all explants and plant growth regulator treatments produced compact callus in their study [14].

The impact of culture media towards callus formation and consistency was also reported previously by Avilés *et al*. following their studies in *Juglans regia* L. in which they testified that calli produced from the explants cultured on MS medium were more friable and had less browning effects [15]. On the

other hand, those cultured on WPM medium were more likely to appear nodular. Nevertheless, no generalization could be made in terms of plant response towards culture media factor since morphogenic capacity of the induced calli may vary depending on the species [15]. In contrast with the study by Behbahani *et al*. [16], the greatest callus induction percentage (90%) in *B. racemosa* from leaf explants incubated at 25 °C under dark condition was obtained from the treatment of 2.0 mg/L 2,4-D in WPM medium and they found that the scores were relatively lower in MS medium (13%–48%). Therefore, it has been verified that the contents of the nutritive media influence the morphogenic responses of the species being cultured and the explants used as well.

In terms of time taken for the callus to be induced, generally WPM medium exerted faster induction response in which 11 out of 13 treatments induced the formation of callus as early as during first week of culture (Table 2). In contrast to MS medium, the treatment consisted of 2,4-D hormone alone at the concentration of 1.0–2.0 mg/L resulted in a delayed response in the onset of callus formation (Table 1). The superiority of WPM medium in producing earlier callus induction response was previously reported by Behbahani *et al*. as well wherein it took three weeks for the callus to be produced in leaf explants of *B. racemosa* as compared to other media which required five weeks [16]. On the contrary, Avilés *et al*. reported that callogenesis in *Juglans regia* L. (walnut) started a week earlier in MS medium than in WPM medium [15]. Upon statistical analysis, in terms of callus induction percentage, the effect of different culture media was found to be statistically insignificant with the value of $P > 0.05$. Nevertheless, the difference in culture media exerted significant effects towards callus morphology and fresh weight ($P < 0.05$).

More further studies are potentially conducted to examine the impacts of medium components towards callus morphogenic responses since there are some great differences in the content of macronutrients and micronutrients available in different basal culture media. For instance, there are a great amount of potassium nitrate (1 900 mg/L) found in MS medium while the component is absent in WPM medium. In addition to that, there are nutrients which are available at a relatively higher amount than those in WPM medium and *vice versa*. Ammonium nitrate and calcium chloride for instance are found much higher in MS medium (1 650 mg/L and 440 mg/L respectively) than in WPM medium (400 mg/L and 96 mg/L respectively). Similarly, there are some nutrients which are only found in WPM medium while they are lacking in MS medium such as calcium nitrate, potassium sulfate and mangan sulfate [17]. The differences in the compositions of such nutrients are likely to be the factor which influence the responses of explants of each plant species towards the formation of callus.

The effects of plant growth regulators and culture media on the induction and formation of calli from endosperm of *B. racemosa* had been elucidated from this study. The findings gathered are useful for production of calli, which is required for plant regeneration studies, somatic embryogenesis or it may function as a starting point for establishing cell suspension cultures, plant bioreactor and bioactive compounds studies in the species.

Conflict of interest statement

We declare that we have no conflict of interest.

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