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Effects of 2,4-D and Kinetin on Callus Induction of *Barringtonia racemosa* Leaf and Endosperm Explants in Different Types of Basal Media

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Abstract: *Barringtonia racemosa*, was a medicinal plants that widely used in traditional practice to treat various ailments and even its young leaf were eaten raw. Due to its healing properties, the tree was intensively harvested without replacement planting effort and *in vitro* technique can be one of the ways to propagate them in the field. The purpose of this study was thus to develop a standardized basic protocol to induce a high frequency of callus induction. The effects of 2,4-Di-chlorophenoxy-acetic acid (2,4-D) and 6-furfuryl aminopurine (kinetin) on callus induction percentage (CI%) of *B. racemosa* leaf and endosperm explants in Murashige and Skoog medium, Lloyd and McCown medium, Murashige and Skoog (Modified) medium were evaluated. The following three basal media were added: 30 g L^{-1} of sucrose, 2.5 g L^{-1} phytagel and combination of (2,4-D) with kinetin in various concentrations (0.5,1.0,1.5 and 2.0 mg L^{-1}). The highest CI% was shown by *B. racemosa* endosperms culture, with 100% callusing in MS medium added with 1.5 mg L^{-1} 2,4-D and 0.5 mg L^{-1} kinetin. The callus was friable, beige and grew intensively. In leaf culture, the highest CI% was 93.33% with friable, beige colour callus in MS medium supplemented with 2.0 mg L^{-1} of 2,4-D. Results indicated that 2,4-D and kinetin with certain combinations in MS medium gave better callusing compared to other basal media. These results provided a basis for the optimization of callus culture protocol to develop a reproducible of plantlet, inducing callus for somatic embryogenesis and secondary metabolite production.

Key words: *Barringtonia racemosa*, 2,4-Di-chlorophenoxy-acetic acid, kinetin, callus induction

INTRODUCTION

Barringtonia racemosa belong to the Lecythidaceae family (Berkov *et al*., 2000), which can be found in tropical Asia (Mori 2007). The leaf were used to treat itch, chicken-pox or rheumatism febrifuge besides its were consumed as traditional vegetable among people in the Southeast Asia (Burkhill, 1966; Samy *et al*., 2005). Khan *et al*. (2001) reported the ethanol extracts of *B. racemosa* roots exhibited antibacterial activity against *Bacillus cereus* and also *Salmonella typhi.* Besides that the aqueous bark extract of *B. racemosa* has antinociceptive activity with no side effects or toxicity when treated in normal rats (Dearniyagala *et al*., 2003). In India, the previous folk medicine practices used the seeds of *B. racemosa* to treat certain tumours in a few remote villages (Thomas *et al*., 2002). *B. racemosa* leaf chloroform extract also showed anti-inflammatory properties without cytotoxic effect plus a good correlation with antioxidant activity of the same sample (Behbahani *et al*., 2007). Hussin *et al*. (2009) found that the leaves, sticks and barks ethanol extracts showed antifungal activity especially to inhibit the growth of *Fusarium* sp., Previous research also revealed that *Barringtonia* species contains saponin, alkaloids, flavonoids, sterols, triterpenoids, tannins, diterpenoids, polyphenols and cyanogenic constituents (Berkov *et al*., 2000; Hasan *et al*., 2000; Khan and Omoloso, 2002).

Due to its healing properties, it was rapidly harvested without replacement of the tree by conventional propagation as well as by *in vitro* technique. *B. racemosa* can be propagate through seeds and cutting (Jansen and Cardon, 2005) but it had their own disadvantage such as low viability, low germination rate and delayed rooting of the seedlings (Thomas and Maseena, 2006). By *in vitro* technique, one of the methods to develop a reproducible of plantlet was through callus because it was the most suitable material used for genetic transformation in plant (Michel *et al*., 2008), increasing secondary metabolites

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production (Mihaljevic *et al*., 2002) and induction of somatic embryogenesis (Maciel *et al*., 2010).

Explant selection were very crucial in callus induction because explant response is highly genotype dependent, so that for any given species, not all different types of explant were suitable to induce embryogenic callus (Trigiano and Gray, 2005). Ideal explants must contain a lot of competent cells likes' young leaf because it could be easily adjusted in *in vitro* conditions (Yan *et al*., 2009). Endosperm also a suitable explants since it lacks any differentiation and consists mostly of parenchymatous cells (Chawla, 2002). Besides that, to get callus grow rapidly, three different basal media were screened, which is Murashige and Skoog (1962) Lloyd and McCown (1980) (Modified). Under *in vitro* propagation, almost plants respond to Murashige and Skoog (1962) medium but not all species will showed optimum growth because it may not tolerate for high salt content in it (Trigiano and Gray, 2005). Murashige and Skoog (1962) medium are highly concentrated in macronutrients compare to Lloyd and McCown (1980) medium especially differences with the nitrogen and calcium concentrations (Michel *et al*., 2008). Lloyd and McCown (1980) medium with low salt content are quite commonly used for woody plants which have salt sensitivity.

Balancing level auxin and cytokinin in the basal medium very important because it governs the dedifferentiation or differentiation mechanisms of explant (Bourgaud *et al*., 2001). A 2,4-Di-chlorophenoxy-acetic acid (2,4-D) is commonly auxin applied to induce callus growth because it can revert cells in the explant to a dedifferentiated state and begin to divide (George *et al*., 2008). Kinetin under the group of cytokinin was added to stimulate cell division and control morphogenesis of the cells (George *et al*., 2008).

Thus, in order to develop a reproducible and an efficient medium for callus induction, the effects of leaf and endosperm explants, basal media, 2,4-Di-chlorophenoxy-acetic acid (2,4-D) and kinetin combination on callus induction of *B. racemosa* as a starting point for the development of *in vitro* plantlet regeneration of this species were evaluated.

MATERIALS AND METHODS

Plant materials: The leaves and young fruits were obtained from the Herb Garden in Malaysian Agricultural Research and Development Institute (MARDI) Jerangau, Kuala Terengganu, experimental station.

Sterilization of explants material: The explants were washed with Teepol® and distilled water and then rinsed under running tap water for an hour. In the sterile vessels, the explants were shaken in 70% v/v ethanol in 30 sec for leaf and 3 min for the fruits. Then, all the explants were placed for 20 min in 20% v/v commercial sodium hypochlorite added with a few drops of Tween 20® with vigorous mixing. Finally, the explants were rinsed three times with sterile distilled water. The endosperm and leaf were cut into small pieces (5×5 mm).

Preparation of basal media: Murashige and Skoog (1962) and Lloyd and McCown (1980) (Modified) solid medium supplemented with of 2,4-D (Duchefa Biochemie, Netherlands) with/without Kinetin (Duchefa Biochemie, Netherlands) at various concentration (0–2.0 mg L^{-1}) and sucrose (Duchefa Biochemie, Netherlands) 3.0% (w/v) was added as carbon source. The medium was autoclaved (Tommy, Japan) at 121°C for 20 min after adjusting the pH to 5.8 and Phytagel (Sigma, USA) was added to the solution at the rate of 2.5 g L^{-1} (0.25% w/v). All the cultures were incubated in incubation room at 25±2°C in the dark and the morphologies with the percentage of Callus Induction (CI) were observed and recorded within four weeks.

Histology preparation of callus: Callus for histological studies were taken from the four weeks old cultures. The tissues were fixed with 50.0% Formalin Acetic Acid (FAA) for 48 h at room temperature. After dehydrating in series of ethanol (30 and 50%) followed by ascending Tertiary Butyl Alcohol (TBA) (60, 70, 85, 95 and 100%) and lastly by parraffin oil, they were embedded in paraffin wax at 60°C and cut into 10 μm section (RM 2245, Leica, Germany) rotary microtome. The sections were transferred and affixed to glass slides. After staining with safranin and fast green, mounting in Canada balsam and covering with a cover glass, they were observed and photographed under a fluorescent microscope (DM 5000 B, Leica, Germany) in a bright-field contrast.

Data analysis: Data were collected after 4 weeks of culture. There were five replicates with 10 explants in each replicate assessed. The Callus Induction Percentage (CI%) was calculated as follows:

$$\text{Callus Induction Percentage (CI\%)} = \frac{\text{No. of explant with callus}}{\text{Total No. of explant}} \times 100$$

Statistical analysis was done by ANOVA and Duncan's Multiple Ranges Test (DMRT) using the Statistical Package for Social Science (SPSS) Programme. Data are expressed as mean of three determinations±SD.

RESULTS

Effect of 2,4-D and kinetin in Murashige and Skoog (1962) from the leaf explants: The percentage of callus induction ranged from 40.0 to 93.9% with various degree of callus growth (Table 1). The highest percentage of CI (93.33%) in MS (1962) was from the treatment of 2,4-D at 1.0 mg L^{-1} with intensively growth callus compare to other treatments (Fig. 1a). The additions of kinetin also induce the callus growth but reduce the rate of callusing and gave soft and watery callus. These results showed that 2,4-Dalone was sufficient for callus induction of *B. racemosa* leaf in MS (1962).

Effect of 2,4-D and kinetin in Murashige and Skoog (1962) (Modified) from the leaf explants: From the Table 1, the highest CI percentage was from the treatment 0.5 mg L^{-1} of 2,4-D (90.0%) but callus grow intensively at the concentration 1.5 mg L^{-1}. Increasing the concentration of 2,4-D till 2.0 mg L^{-1} had reduced percentage of callus induction and gave compact and brownish callus. Adding kinetin resulted soft texture of callus and lower the CI percentage.

Effect of 2,4-D and kinetin in, Lloyd and McCown (1980) from the leaf explants: The highest percentage of callus induction (90.0%) from the combination of 1 mg L^{-1} of 2,4-D with 0.5 mg L^{-1} of kinetin and also from the treatment 2 mg L^{-1} of 2,4-D (Table 1). In both treatments, only treatments of 2 mg L^{-1} of 2,4-D gave friable and beige callus. All the treatments applied in Lloyd and McCown (1980) medium showed callus growth but the induced callus were slightly grow with either soft or compact texture.

Effect of 2,4-D and kinetin in Murashige and Skoog (1962) from the endosperm explants: In the 2,4-D treatment only, all explants showed callus growth (100% of CI) (Table 2). The callus grow rate however decrease with increasing concentration of 2,4-D. The addition of kinetin into 2,4-D had increase the frequency of callus induction up to 100% only in the treatment 1.5 mg L^{-1} of 2,4-D plus 0.5 mg L^{-1} kinetin (Fig. 1b) and 1.0 mg L^{-1} of 2,4-D plus 1.0 mg L^{-1} kinetin.

Effect of 2,4-D and kinetin in Murashige and Skoog (1962) (Modified) from the endosperm explants: The percentage of callus induction ranged from 96.0 till 100% and most of the treatments gave friable texture callus and grow vigorously (Table 2). Between all the treatment, rapid growing of callus with friable, beige colour was showed in the treatment 0.5 mg L^{-1} 2,4-D. The addition of kinetin in concentration at 0.5 mg L^{-1} will increase the callus growth and gave friable, beige colour.

Effect of 2,4-D and kinetin in, Lloyd and McCown (1980) from the endosperm explants: It was observed that the highest growth rate of callus was obtained from the treatment 1.0 mg L^{-1} 2,4-D plus 0.5 mg L^{-1} kinetin (100%) and also 1.0 mg L^{-1} 2,4-D plus 1.0 mg L^{-1} kinetin (100%)

Table 1: Effects of the 2,4-D and kinetin concentration in the basal mediums on the callus induction from leaf explants of *B.racemosa*

Plant growth regulator		Murashige and Skoog (1962) Medium			Murashige and Skoog (1962) (Modified)			Lloyd and McCown (1980) Medium		
2,4-D (mg L^{-1})	K (mg L^{-1})	Callus induction (%)	Callus growth	Callus texture	Callus induction (%)	Callus growth	Callus texture	Callus induction (%)	Callus growth	Callus texture
0.0	0.0	0.0±0.00^{a}	NC	-	0.00±0.0^{a}	NC	-	0.00±0.0^{a}	NC	-
0.5	0.0	83.0±0.58^{d}	+ +	Compact	90.0±1.00^{e}	++	Friable	16.7±2.89^{a,b}	+	Soft
1.0	0.0	93.3±1.15^{d}	+ + +	Friable	73.3±0.58^{e}	++	Friable	0.0±0.00^{a}	NC	-
1.5	0.0	56.7±3.21^{c}	+ +	Friable	70.0±2.65^{d,e}	+++	Friable	0.0±0.00^{a}	NC	-
2.0	0.0	90.0±0.00^{d}	+ + +	Friable	53.3±1.50^{c,d}	++	Compact	90.0±0.00^{c}	+ +	Friable
0.0	0.5	0.0±0.00^{a}	NC	-	0.0±0.00^{a}	NC	-	0.0±0.00^{a}	NC	-
0.5	0.5	23.0±0.57^{b}	+	Soft	63.3±1.53^{c,e}	+	Friable	36.7±4.04^{b}	+	Compact
1.0	0.5	56.6±1.58^{c}	+	Soft	0.0±0.00^{a}	NC	-	90.0±1.00^{c}	+	Soft
1.5	0.5	40.0±2.00^{b,c}	+	Soft	46.7±0.58^{b}	+	Soft	0.0±0.00^{a}	NC	-
0.0	1.0	0.0±0.00^{a}	NC	-	0.0±0.00^{a}	NC	-	0.0±0.00^{a}	NC	-
0.5	1.0	40.0±1.00^{b,c}	+	Friable	0.0±0.00^{a}	+	-	63.3±2.30^{c}	+	Soft
1.0	1.0	46.7±1.15^{c}	+	Friable	30.0±1.00^{b}	+	Soft	13.3±2.30^{a,b}	+	Soft
0.0	1.5	0.0±0.00^{a}	NC	-	0.0±0.00^{a}	NC	-	0.0±0.00^{a}	NC	-
0.5	1.5	0.0±0.00^{a}	NC	-	0.0±0.00^{a}	NC	-	0.0±0.00^{a}	NC	-
0.0	2.0	0.0±0.00^{a}	NC	-	0.0±0.00^{a}	NC	-	0.0±0.00^{a}	NC	-

Data were recorded after 4 weeks of culture incubation. Value represent the mean±SD following the same letter within columns are significantly different, according to Duncan's Multiple Range Test ($p<0.05$). NC: No callus obtained,-: Indicate no response, +: Slight callusing. ++: Considerable callusing, +++: intensive callusing

(Table 2). It was found that although the concentration of kinetin was higher than 2,4-D, the callus still can be induced from the endosperm explants. In the treatment of 0.5 mg L^{-1} 2,4-D plus 1.5 mg L^{-1} kinetin, the callus growth still high with beige, friable texture. This showed that callus can still grow although the concentration of 2,4-D was lower than kinetin.

Histology observation of the callus: Histological observations were carried out to determine the callus origin from the leaf and endosperm explants. Both the leaves and endosperm showed densely stained callus cells with the callus tissue structure showed actively dividing undifferentiated cells. Figure 2a showed the origins of the callus (CO) were expected to be from mesophyll cells of leaf besides the structure of epidermis, mesophyll and vein were destructed and the callus tissues were found scattered all over the meristematic region. The anatomy study also showed the structure of parenchyma cells which is isodiametric shaped cells, thin wall type, with masses of loose structure with large size. From the Fig. 2b, the origins of callus (CO) were from the parenchyma cells of endosperm.

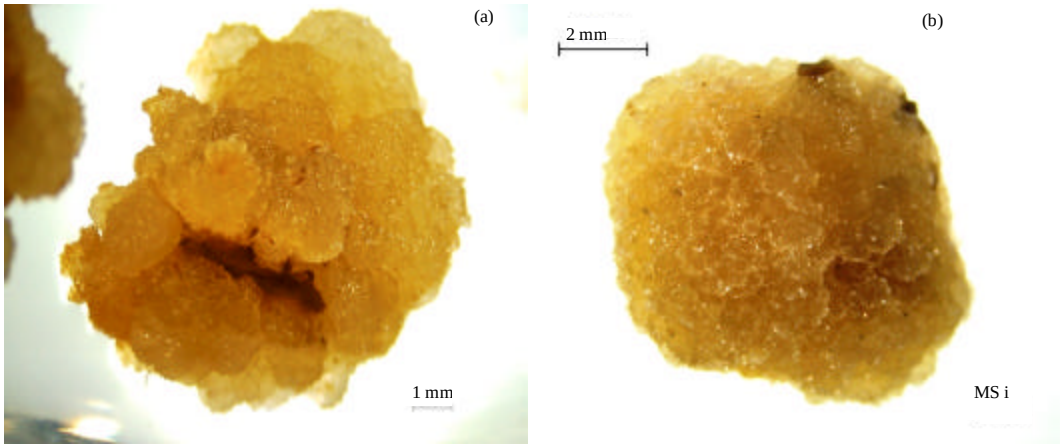

Fig. 1(a-b): Callus induction from leaf segments cultures of *B.racemosa* after 4 weeks of incubation in Murashige and Skoog (1962). (a) A callusing leaf segment containing 1.0 (mg L^{-1}) of 2,4-D; Bar 1 mm, (b) A callusing endosperm segment supplemented with 1.5 mg L^{-1} of 2,4-D and 0.5 mg L^{-1} kinetin, Bar 2 mm

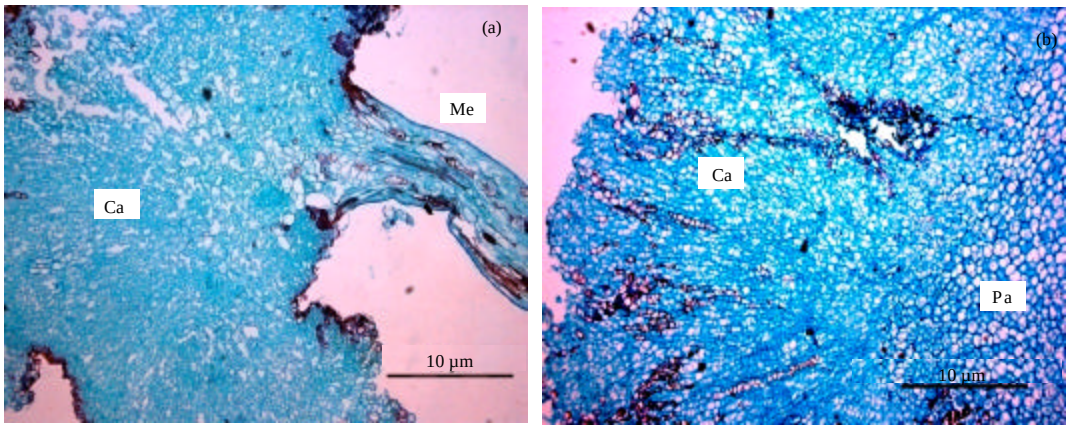

Fig. 2(a-b): Histological observation of callus induction from *B. racemosa.* (a) *B. racemosa* callus which is still attached to the leaf explants, exhibiting remnants of the mesophyll cells of the explants with callus cells formation from the leaf discs, (b) *B. racemosa* callus on the left, which can be seen to radiate from the endosperm explants, exhibiting remnants of the parenchyma cells on the right. Bar, 10 µm, Me: Mesophyll, Ca: callus, Pa: Parenchyma

Table 2: Effects of the 2,4-D and kinetin concentration in the Murashige and Skoog 1962 basal medium on the callus induction from endosperm explants of *B. racemosa*

Plant growth regulator		Murashige and Skoog (1962) Medium			Murashige and Skoog (1962) (Modified)			Lloyd and McCown (1980) Medium		
2,4-D (mg L^{-1})	K (mg L^{-1})	Callus induction (%)	Callus growth	Callus texture	Callus induction (%)	Callus growth	Callus texture	Callus induction (%)	Callus growth	Callus texture
0.0	0.0	0.0±0.0^{a}	NC	-	0.0±0.00^{a}	NC	-	0.0±0.00^{a}	NC	-
0.5	0.0	93.3±1.15^{c,d}	++	Friable	98.0±0.45^{b}	++++	Friable	100.0±0.00^{c}	++	Soft
1.0	0.0	96.7±0.58^{d}	++	Friable	100.0±0.00^{b}	+	Friable	100.0±0.00^{c}	++	Soft
1.5	0.0	76.7±0.58^{b,c}	+	Friable	96.0±0.55^{b}	++	Friable	96.7±0.58^{c}	+	Soft
2.0	0.0	100.0±0.0.0^{d}	+	Friable	100.0±0.00^{b}	+++	Friable	96.7±0.58^{c}	+	Friable
0.0	0.5	0.0±0.0.0^{a}	NC	-	0.0±0.00^{a}	NC	-	70.0±2.64^{b}	NC	-
0.5	0.5	96.7±0.58^{d}	++	Compact	98.0±0.45^{b}	++++	Soft	100.0±0.00^{c}	+++	Compact
1.0	0.5	60.0±3.60^{b}	++	Friable	98.0±0.45^{b}	+++	Soft	100.0±0.00^{c}	+++	Friable
1.5	0.5	100.0±0.00^{d}	+++	Friable	98.0±0.45^{b}	++	Friable	93.3±0.58^{c}	++	Friable
0.0	1.0	0.0±0.00^{a}	NC	-	0.0±0.00^{a}	NC	-	96.7±0.58^{c}	+	Friable
0.5	1.0	100.0±0.00^{d}	+++	Compact	100.0±0.00^{b}	+++	Soft	100.0±0.00^{c}	++	Soft
1.0	1.0	100.0±0.00^{d}	+++	Friable	97.5±0.50^{b}	+++	Friable	100.0±0.00^{c}	+++	Friable
0.0	1.5	0.0±0.00^{a}	NC	-	0.0±0.00^{a}	NC	-	0.00±0.0^{a}	NC	-
0.5	1.5	100.0±0.00^{d}	+++	Friable	100.0±0.00^{b}	+++	Soft	100.0±0.00^{c}	+++	Friable
0.0	2.0	0.0±0.00^{a}	NC	-	0.0±0.00^{a}	NC	-	0.00±0.0^{a}	NC	-

Data were recorded after 4 weeks of culture incubation. Value represent the mean±SD following the same letter within columns are significantly different, according to Duncan's Multiple Range Test ($p<0.05$). NC: No callus obtained, -: Indicate no response, +: Slight callusing, ++: Considerable callusing, +++: Intensive callusing, ++++: Vigorously callusing

DISCUSSION

In this study, the callus induction of *B. racemosa* from the leaf and endosperm explants in three different basal media; Murashige and Skoog (1962) (Modified) and Lloyd and McCown, 1980) medium supplemented with 2,4-D and kinetin at various concentrations was examined. Most culture from woody plant species tends to react on Lloyd and McCown (1980) because this species are less tolerant on high salts content to other media. However from the results, Murashige and Skoog (1962) medium was most suitable as a callus induction medium compare to other two basal media tested in order to obtain high rate of callus grow from the leaf and endosperm explants. The differences in callusing grow rate between the basal media might be due to their formulation where it is highly concentrated macronutrient in Murashige and Skoog (1962), while in Lloyd and McCown (1980), it contain less of macronutrients (Abdellatef and Khalafallah, 2008). The lowest rate of callus grow from the leaf explants obtained in Lloyd and McCown (1980) medium can be due to the deficiency of phosphorous and nitrogen in the basal medium (Michel *et al*., 2008). Besides that, according to Jansen and Cardon (2005), the *B. racemosa* grows well under slightly saline conditions or on beaches near the high water level thus it grow well when cultured on Murashige and Skoog (1962) basal media. From all the results obtained, it showed that the composition of each basal medium can influence the rate of callus growth and its morphology.

Between the explants, the average percentage of CI (%) was higher in endosperms compare to the leaves, but both could exhibit high percentage of callus induction in the suitable treatments and these showed that they were fairly good sources of explants for callus induction.

In all treatments, it was found that basal media without plant growth regulators and contain only kinetin did not induce any callus growth. Combination of 2,4-D and kinetin in various concentrations in three types of basal media had varying effect in callus growth for both species and explants. Comparison between all treatment applied showed that 2,4-D is essential for callus induction and addition of kinetin in certain concentration will increase the callus growth in endosperm culture. This whole outcome showed that 2,4-D was essential for callus induction and kinetin was useful to promote callus growth.

Although the percentage of CI can reach up to 100%, the callus were distinguishable based on the morphological appearance. According to Trigiano and Gray (2005) usually rapid growth and a light colour callus indicate a healthy culture and a friable callus of crumbly appearance is very suitable for breaking up, either for sub-culturing or to produce a suspension culture.

Histological observation of callus from leaf and endosperm *B. racemosa* did not show any embryogenic response because although the cells of the callus were actively dividing besides the size of the cells varied differently with each other. According to Maciel *et al.* (2010), Narciso and Hattori (2010) and Yang *et al.* (2009), non-embryogenic calli appeared as large, loosely adjoined oval cells and spherical cells, with shape and sizes of cells which was composed of these tissues varied greatly. It is believed that calli have different abilities for somatic embryogenesis as their

cells are in different conditions and have different characteristics (Shang *et al*., 2009). The characteristic of embryogenic structure of callus according to Shang *et al*. (2009), Sane *et al*. (2006) and Schwendiman *et al*. (1988) were based on accumulation of starch as indicator for development of the tissues towards embryogenesis, displayed similar cellular morphology, lack of intercellular spaces as well as some nodular structures on the surface, contained dense cytoplasm and appeared to be similar to meristematic cells. Endosperm cells are known to be triploids, thus if these callus can be differentiated, the embryoids obtained may be triploids. This will be very interesting for future investigation.

CONCLUSION

Endosperms and leaf explants showed various extent of callus growth in all media applied and in all treatments. 2,4-D was suitable for callus induction of which gave lighter colour and friable texture. Addition of kinetin was efficient in callus induction for endosperm culture but did not promote the callus growth in leaf. Examination of the anatomical sections showed that callus obtained from leaves and endosperms of *B. racemosa* derived from the mesophyll spongy cells and parenchyma cells, respectively. The cells were not the embryogenic type because they lack intercellular spaces and contain dense cytoplasm.

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