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Full Length Research Paper

Plant regeneration from leaf explants of *Barringtonia racemosa*

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A protocol for *in vitro* propagation from leaf explants of *Barringtonia racemosa* is reported. Calli were aseptically raised by placing surface sterilized leaf explants on Woody Plant Medium (WPM) supplemented with different concentrations of 2, 4-dichlorophenoxyacetic acid (2,4-D). On the shoot induction medium, the callus induced on the WPM medium containing 2 mg/L (w/v) KIN+0.2 mg/l (w/v) IBA and 2 mg/L (w/v) of KIN +0.4 mg/L(w/v) of NAA was the most effective, providing high shoot regeneration frequency of 85.6 and 76.5% respectively. In addition, the highest number of shoots produced was 8.2 and 6.3 shoots per explant respectively in the medium containing the mentioned phytohormones. The good rooting percentage 62 and 5.6% roots per shoot were achieved on WPM medium supplemented with 3g/L (w/v) of active charcoal and 0.8 mg/L (w/v) of IBA. Approximately 96% of the *in vitro* raised plantlets with well developed shoots and roots were survived after transferring to mixed soil and grown well in the glass house.

Keywords: *Barringtonia racemosa*, rooting, shoot regeneration.

INTRODUCTION

Barringtonia racemosa (family Lecythidaceae) is a moderate sized, tropical evergreen tree found in the West Coast of India, Sundarbans, Assam and Andaman Islands (Thomas et al., 2002). It is commonly used in traditional medicine in Malaysia and locally known as 'putat'. The putat fruits are used to treat cough, asthma and diarrhea while its viable seeds are aromatic and useful in colics, jaundice and ophthalmic. The pharmacological uses of *B. racemosa* indicate it to be a rich source of phytomedicine. Previous investigation on the bioactivity of *B. racemosa* has shown that the ethanol extract of *B. racemosa* barks has anti- tumor property and toxicity in mice (Khan et al., 2001). whilst the ethanol extract of the plant leaves displayed cytotoxicity against the HelA (human cervila carcinoma) cell line with a IC_{50} value of 10 g/ml (Mackeen et al., 1997) . The aqueous extract isolated from the stem bark of *B. racemosa* has been displayed to have antinociceptive and toxicological effect on rats (Thomas et al., 2002) while an ethanol extract of the roots of *B. racemosa* provided two novel clerodane diterpenoid nasimalun A and B by NMR and MS analysis (Khan et al., 2000) . The ability to regenerate *B. racemosa* plant in *in-vitro* would enable the rapid asexual propagation of this important agricultural plant. Conventional breeding methods are extremely time consuming because they depend on cross pollination, seed germination and selection as well as vegetative regeneration. Modern plant biotechnology and genetic engineering has the potential to reduce the time needed for traditional breeding. To our knowledge, regeneration of *B. racemosa* using leaf explant has not been reported so far. Therefore, in this study, we attempted to develop an efficient protocol for the rapid regeneration of *B. racemosa* from leaf explants in order to ensure its availability for use in traditional medicine.

MATERIALS AND METHODS

Plant material

Young leaves (5-7x 3-5 cm) of *B. racemosa* were collected from 2-month old seedlings grown in the greenhouse at the University Putra Malaysia, Serdang, Selangor, Malaysia. These leaves were

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surface sterilized with 70% (v/v) ethanol for 1 min, followed by 20% (v/v) solution of commercial bleach (Clorox, 5.25% (w/v) of sodium hypochlorite) added with one drop of polyoxyethylene sorbitan monoleate for 30 min, then rinsed four times with sterile distilled water. For callus induction, the explants were aseptically cut into small pieces. Sterile explants with the size of 0.5 ×0.5 cm were aseptically placed on Woody Plant Medium (WPM) supplemented with different combinations of kinetin (KIN, 1-3 mg/L) and 2,4-dichlorophenoxyacetic acid (2,4-D, 0.5-3 mg/L), containing 30 g/L (w/v) of sucrose and 7 g/L (w/v) agar (Sigma, Germany). pH of medium was adjusted to 5.75 by addition of 1N NaOH. The medium was then autoclaved at 121˚C for 15 min. The explants were incubated at 25 ±1˚C under dark condition for 35 days. After 5 weeks of culture, the percentage of explants producing callus was recorded. The observation was made on the morphology of callus formed in different phytohormones tested.

In vitro shoot induction

Well- proliferated calli derived from the leaf segments five weeks after culture on the proliferation medium (first passage) and without traces of mother explants were used for regeneration studies. Approximately two grams of fresh callus were placed on the WPM medium containing 30 g/L (w/v) of sucrose, 7 g/L (w/v) Gelrite agar and plant growth regulators. Plant growth regulators applied in this study were combinations of BAP (0 - 3 mg/l) and IBA (0 - 0.6 mg/l) or NAA (0 - 0.6 mg/l; Table 2). Data on the mean number of transferable shoots per explant and percentage of shoot formation were observed after seven weeks of culture. Each experiment consisted of 5 replicates and was independently repeated three times. All cultures were incubated at 25 ± 1˚C under light (12000 lux) condition.

In vitro rooting induction

For induction of roots, *in vitro* regenerated shoots (4.1 - 4.6 cm length with 7 - 8 leaves) were individually transferred to WPM basal medium supplemented with 30g/l (w/v) of sucrose, 3 g/l (w/v) of active charcoal and 7 g/l (w/v) of Gelrite agar and plant growth regulators. Plant growth regulators supplied in this study were IBA (0 - 1 mg/L) and NAA (0 - 1 mg/L) or IBA (0 - 1 mg/L). The frequency of rooting and average root number per shoot was recorded. The pH of all the media was adjusted to 5.8 prior to autoclaving at 121°C, for 20 min. All cultures were incubated at 25 ± 1°C, under a 16 h photoperiod. Two month after culture on rooting medium, root length was measured by taking out the rooted plantlets carefully from the media and plantlets with vigorously growing roots were transferred to mixed soil and acclimatized in the greenhouse. Each treatment consisted of 10 explants and was done in triplicate. Data expressed in means ± standard deviation (SD) were used for statistical analysis.

RESULTS AND DISCUSSION

Callus induction of *Barringtonia raceomosa*

Among the three established basal media tried (MS, B5, WPM), WPM was most suitable for callus initiation from leaf explants. Hence, in all experiments only WPM basal medium was used. Callus was optimally induced from leaf segments derived from 2 months old seedlings within 21 - 28 days of inoculation in all treatments containing 2,4-D which applied individually or in combination with KIN. Callusing was initiated at the cut ends of the explants and eventually extended all over the explants. As shown in the results (Table 1), 2 mg/L 2,4-D was found to produce significantly higher callus (90%) as compared to other treatments. Although, auxin is able to promote the growth of callus(site some reference in support of this statement and it will be of more value if you site some recent studies), however according to Wernicke and Milkovits (1987), high concentrations of 2,4- D were able to inhibit callusing of basal segments and it had effect as the herbicide. The addition of KIN reduced callus induction in treatments containing 2,4-D means that the KIN did not promote the growth of callus. The increasing concentration of KIN may lengthens the period needed for the induction of callus. The callus which induced from leaf explants on WPM basal medium containing 2 mg/L (w/v) of 2,4- D was pale yellow and friable. The addition of KIN up to 2 mg/L (w/v) to the medium containing different concentrations of 2,4-D leads to the production of callus which is compact instead of friable. If more than 2 mg/L (w/v) of KIN is added, the callus which is obtained is hard callus. The callus proliferation was best observed on a WPM basal medium containing 2 mg/L (w/v) of 2,4-D.

Shoot bud regeneration from callus cultures

Calli derived from leaf segments, after one passage in culture on proliferation medium were used for shoot regeneration. The percentage of calli showing shoot regeneration was the highest (85.6 and 76.5%) on medium containing 2 mg/L (w/v) of BAP + 0.2 mg/l IBA, and 2 mg/L (w/v) of BAP + 0.4 mg/L (w/v) of NAA respectively (Table 2). Regeneration of green shoot buds occurred in these calli after the 31 and 33 days of culture and maximum number of shoot bud emergence occurred in the seventh week. These shoot buds elongated and attained an average height of 4.6 and 4.1 cm in a WPM basal medium containing the mentioned phytohormones. Referring to Table 2, the addition of NAA up to 0.4 mg/L (w/v) and IAA up to 0.2 mg/L (w/v) into the culture medium enhanced the mean number of transferable shoots per explant and percentage of shoot formation. Light is required for normal differentiation of shoot. Light is believed to have phytochrome effect on photomorphogenesis in plants. Callus could grow on medium containing cytokinin but did not differentiate in the WPM basal medium supplemented with cytokinin without auxin. Hence, exogenous auxin was needed for multiple shoot formation of *B. racemosa.* Both cytokinin and auxin were required for shoot plant regeneration. The role of BAP seems essential for regeneration. Similarly, for *Dianthus chinensis* plant regeneration from leaf explants has been reported to required WPM basal medium with BAP in combination with NAA (Jethwani and

Table 1. Percentage of callus induction, days of callus induction and morphology of callus formed from *B. racemosa* leaf explants on WPM medium supplemented with different combinations 2,4-D and KIN supplied after 35 days of cultures at 25 ± 1 under dark condition.

Phytohormones supplied in WPM basal medium(mg/L)		Days of callus induction	% Callus induction	Morphology of callus formed from leaf explants
2,4-D	KIN			
0	0	-	NC	-
0.5	0	21 ± 2	34±2.0	Pale yellow, friable
1	0	21± 2	65±2.0	Pale yellow, friable
2	0	20± 1	90±2.2	Pale yellow, friable
3	0	21± 2	75±1.6	Pale yellow, friable
0	1	-	NC	-
0.5	1	24 ±2	27±1.1	Pale yellow, compact
1	1	23± 2	53±1.4	Pale yellow, compact
2	1	23± 1	72±2.3	Pale yellow, compact
3	1	23± 2	60±1.6	Pale yellow, compact
0	2	-	NC	-
0.5	2	27± 2	25±2.3	Pale yellow, compact
1	2	26± 2	43±2.3	Pale yellow, compact
2	2	26± 2	65±2.4	Pale yellow, compact
3	2	26± 2	52±1.3	Pale yellow, compact
0	3	-	NC	-
0.5	3	29±1	15±1.5	-
1	3	28± 2	34±1.4	Pale yellow, hard
2	3	28± 1	50±2.6	Pale yellow, hard
3	3	28± 2	42±2.3	Pale yellow, hard
Phytohormones supplied in WPM basal medium(mg/L)		**Days of callus induction**	**% Callus induction**	**Morphology of callus formed from leaf explants**
2,4-D	**KIN**			
0	0	-	NC	-
0.5	0	21 ± 2	34±2.0	Pale yellow, friable
1	0	21± 2	65±2.0	Pale yellow, friable
2	0	20± 1	90±2.2	Pale yellow, friable
3	0	21± 2	75±1.6	Pale yellow, friable
0	1	-	NC	-
0.5	1	24 ±2	27±1.1	Pale yellow, compact
1	1	23± 2	53±1.4	Pale yellow, compact
2	1	23± 1	72±2.3	Pale yellow, compact
3	1	23± 2	60±1.6	Pale yellow, compact
0	2	-	NC	-
0.5	2	27± 2	25±2.3	Pale yellow, compact
1	2	26± 2	43±2.3	Pale yellow, compact
2	2	26± 2	65±2.4	Pale yellow, compact
3	2	26± 2	52±1.3	Pale yellow, compact
0	3	-	NC	-
0.5	3	29±1	15±1.5	-
1	3	28± 2	34±1.4	Pale yellow, hard
2	3	28± 1	50±2.6	Pale yellow, hard
3	3	28± 2	42±2.3	Pale yellow, hard

Data indicates mean ± Standard deviation (n=5). NC = No callus formed.

Kothari, 1996). Generally shoot buds will be initiated in medium containing a high ratio of cytokinin to auxin (Hare et al., 1997). BAP has been used extensively in recent years and seemed to be the common cytokinin for tissue

Table 2. The effects of phytohormones (BAP and IBA or NAA) supplied on percentage of shoot regeneration, number of shoots, stem length (cm) and days shoot start to form from *B. racemosa* callus cultures. The number of shoots per callus and stem length (cm) were recorded after 7 weeks of culture on WPM medium (pH 5.8) at 25 C ± 1.

Phytohormones combination In WPM basal medium (mg/L)			Days of shoot start to form	% shoot regeneration	No. of shoot	Stem length(cm)
BAP	IBA	NAA				
0.5	0	0	0	0	0	0
0.5	0.2	0	36 ± 2	42.5 ± 1.4	5.1 ± 1.4	3.2 ± 0.2
0.5	0.4	0	38 ± 1	36.4 ± 1.1	4.0	2.9 ± 0.1
0.5	0.6	0	40 ± 1	22.1 ± 1.2	2.2	2.2 ± 0.1
1	0	0		0		
1	0.2	0	36 ± 1	53.6 ± 1.1	6.3	3.5 ± 0.2
1	0.4	0	37 ± 1	46.4 ± 1.2	5.4	3.3 ± 0.1
1	0.6	0	38 ± 1	35.0 ± 0.9	4.7	2.7 ± 0.1
2	0	0		0		
2	0.2	0	31 ± 1	85.6 ± 2.1	8.2	4.6 ± 0.3
2	0.4	0	35 ± 1	69.4 ± 1.3	7.7	3.8 ± 0.2
2	0.6	0	37 ± 1	45.4 ± 1.4	4.0	3.2 ± 0.2
3	0	0		0		
3	0.2	0	38 ± 1	37.2 ± 1.0	4.3	2.9± 0.1
3	0.4	0	40 ± 1	25.7 ± 0.8	2.5	2.1± 0.1
3	0.6	0	42 ± 1	15.4 ± 0.6	2.4	1.4± 0.1
0.5	0	0	0	0		0
0.5	0	0.2	40 ± 2	35.4 ± 2.0	3.2	3.2 ± 0.1
0.5	0	0.4	38 ± 1	42.4 ± 2.2	4.1	3.9 ± 0.2
0.5	0	0.6	40 ± 2	30.2 ± 1.1	3.5	2.7 ± 0.1
1	0	0		0		
1	0	0.2	37 ± 1	46.6 ± 2.4	4.2	3.7 ± 0.2
1	0	0.4	36 ± 1	51.5 ± 1.9	4.3	3.8 ± 0.1
1	0	0.6	38 ± 1	40.4 ± 2.3	4.2	3.5 ± 0.1
2	0	0		0		
2	0	0.2	35 ± 2	62.4 ± 1.2	5.5	4 ± 0.3
2	0	0.4	33 ± 1	76.5 ± 1.1	6.7	4.1± 0.2
2	0	0.6	37 ± 1	50.6 ± 1.5	4.3	3.8 ± 0.2
3	0	0	0	0		
3	0	0.2	41± 1	21.8 ± 2.2	2.7	2.2 ± 0.1
3	0	0.4	40± 1	38.3 ± 2.4	4.2	3.4 ± 0.1
3	0	0.6	44± 1	16.2 ± 2.3	2.1	1.5 ± 0.1

Data indicates mean ± Standard deviation Data indicates mean ± Standard deviation (n = 5).

culture work (Loiseau et al., 1995).

Rooting of the regenerated shoots

Results on the rootlets induction of the *in vitro* plantlet derived from shoot tip explant cultured in the WPM basal medium supplemented with 3 g/L (w/v) of active charcoal and different levels of IBA, IAA and NAA were summarized in Table 3. The data obtained revealed that only three treatments such as 0.6, 0.8 and 1 mg/L (w/v) of IBA managed to trigger the formation of rootlets from the *in vitro* plantlets within 35 - 48 days. Other treatments tested within the range didn't show any sign of rootlets formation. About 25% rootlets formation occurred in the treatment using 0.6 mg/L (w/v) of IBA while only 62 and 53% was observed in 0.8 and 1 mg/L (w/v) of IBA. The roots were thin and slender with an average length of 3.5 to 4.8 cm. The result obtained revealed that increased concentrations of IBA more than 0.8 mg/L (w/v) brought about a decrease in rooting. Most frequently, root formation is inhibited by the cytokinins and promoted by the auxines. Auxins incorporated into media to induce rooting are IAA (0.1-10 mg/L), NAA (0.05 - 1 mg/L) and IBA (0.5 - 3 mg/L). Sometimes root formation is much

Table 3. The effect of phytohormones alone on percentage of root regeneration, number of root per shoot and root length of *in vitro* derived shoot of *B. racemosa.* The number of root per shoot and root length (cm) were recorded after 8 weeks of culture on WPM basal medium (pH 5.8) at 25 C ± 1.

Hormone Combination In WPM basal medium (mg/L)		Days of root start to form	% root regeneration	No. of root	Root length(cm)
0.2	IAA	-	-	-	-
0.4	IAA	-	-	-	-
0.6	IAA	-	-	-	-
0.8	IAA	-	-	-	-
1	IAA	-	-	-	-
0.2	IBA	-	-	-	-
0.4	IBA	-	-	-	-
0.6	IBA	48 ± 1	25 ± 1.0	2.4 ± 0.2	3.5 ± 0.1
0.8	IBA	35 ± 2	62 ± 2.1	5.6 ± 0.3	5.2 ± 0.2
1	IBA	40 ± 1	53 ± 2.0	4.3 ± 0.3	4.8 ± 0.1
0.2	NAA	-	-	-	-
0.4	NAA	-	-	-	-
0.6	NAA	-	-	-	-
0.8	NAA	-	-	-	-
1	NAA	-	-	-	-

Data indicates mean ± Standard deviation Data indicates mean ± Standard deviation (n = 5).

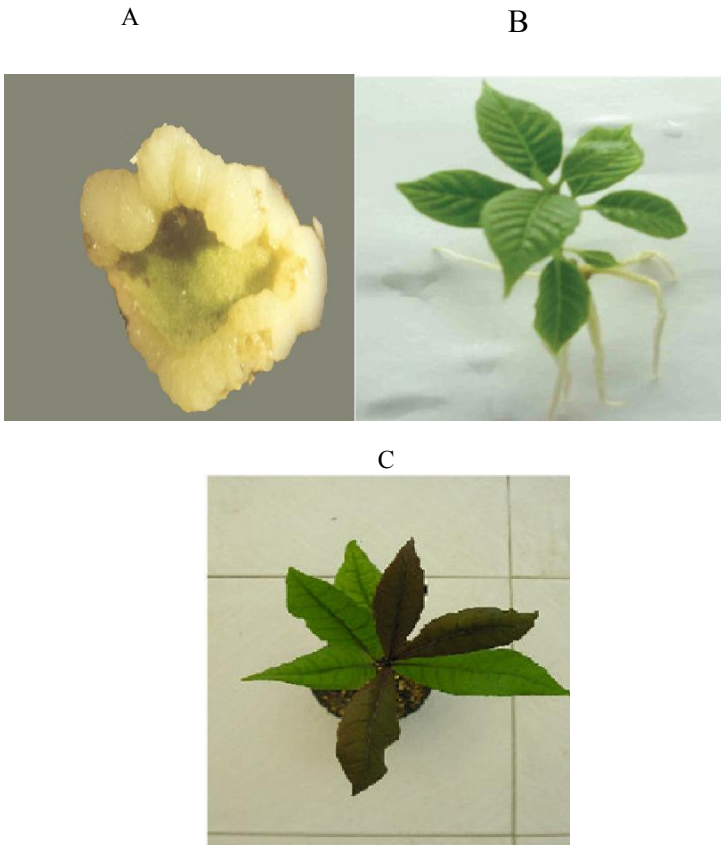

Figure 1. (A): Callus induction on leaf explants on the WPM medium containing 2 mg/L (w/v) of 2,4-D after 5 weeks of culture. (B): Rooted shoot of *B. racemosa* on the medium containing 0.8 mg/L (w/v) of IBA. (C): The plantlet of *B. racemosa* growing in the mixed soil. (Magnification, A=2X; B, C = 1X).

better with one auxin than another. In root elongation phase, too high auxin level in media will inhibit its growth (Thimann, 1977).

Leonardi (2001) and Moreira-Dias et al. (2000) reported that only low concentrations of IBA (0.6 to 1 mg/L) was required for rootlets formation of Grevillea and citrus. According to Juliani et al. (1999), shoot contains high levels of endogenous auxins and the addition of high concentration of exogeneous auxin caused the inhibition of rootlets development. An inhibitory effect of auxin was also observed in apple and *Eucalyptus sideroxylon* when the explants were exposed to a high concentration of IBA (Pawlicki and Welander, 1995). Other treatments tested within the range did not show any sign of rootlets formation. In the *in vitro* plantlets of *B. racemosa* regenerated from the shoot explant, formation of rootlets started to be visible after 35 days of culture in the treatment using 0.8 mg/L (w/v) of IBA while a significant longer period, 40 and 48 days was required for the rootlets formation in 1 and 0.6 mg/L (w/v) of IBA supplied to the WPM basal medium respectively. After six weeks of culture on the rooting medium, the plantlets with normal shoots and roots were transferred to the soil in the greenhouse. During the first two weeks in the greenhouse, air relative humidity > 80% was maintained. Under these conditions over 96% of the plantlets survived and resumed normal growth. In conclusion, we have established a successful *in vitro* system of *B. racemosa* for young leaf explants derived callus.

Experimental design and statistical analysis

The experiments were performed using complete randomized design (CRD) and results were analyzed using one way ANOVA. Experiment was a factorial. If there is a significant different, Duncan Multiple Range Test (DMRT) method at 0.05 (5%) probability significance level will be done in order to determine the significances. Each treatment independently contained 5 replicates with 10 explants per radiate.

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