

Full Length Research Paper

Efficient propagation of an important medicinal plant *Boesenbergia rotunda* by shoot derived callus

Nor Azma Yusuf^{1,2*}, M. Suffian M. Anuar¹ and Norzulaani Khalid¹

¹Centre of Biotechnology for Research in Agriculture, Biotechnology and Bioproduct Research Cluster, Institute of Biological Sciences, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia.

²Faculty of Plantation and Agrotechnology, Faculty of Applied Sciences, University Technology MARA (UiTM) 40450 Shah Alam, Selangor, Malaysia.

Accepted 22 March, 2011

A protocol for high frequency shoot organogenesis and plant establishment from shoot base derived callus was developed for *Boesenbergia rotunda* (L.) Mansf. Kulturpfl. The cultures produced callus in MS medium supplemented with various concentrations of dicamba and 2,4-D. Nevertheless, embryogenic callus only emerged on 2, 4-D augmented MS medium. In addition, the success of callus induction was achieved from meristem derived callus producing 100% of embryogenic callus. Multiple shoot induction was achieved from the surface of the callus after transferring onto MS medium supplemented with BAP or kinetin (shoot induction medium). The highest frequency of callus producing shoots (49.8%) was achieved on MS medium containing 2.0 mg/L BAP. Plantlets with well-developed shoots and roots on MS medium supplemented with 2 g/L activated charcoal were acclimatized and successfully grown in the greenhouse.

Key words: *Boesenbergia rotunda*, plant cell cultures, medicinal plant, organogenesis, Zingiberaceae, micropropagation.

INTRODUCTION

Boesenbergia rotunda (L.) Mansf. Kulturpfl. (Larsen, 1996), is a traditional medicinal plant which belongs to Zingiberaceae family. This species is believed to have originated from India and South-East Asia region. It is believed that it possesses anti-inflammatory effects, provides relief from bacterial dysentery, stomach ache, anti-flatulence and helps to promote appetite. The tubers are widely used as local application for suppression of tumors, swellings and wounds. *B. rotunda* species is beneficial for pharmacological industry with the

presence of flavanoids, essential oil and chalcones which have precious pharmacological properties (Jaipetch et al., 1982; Pandji et al., 1993; Trakoontivakorn et al., 2001). Furthermore, rhizomes contain a potential antipyretic, analgesic, anti-mutagenic, anti-inflammatory and antioxidant enzymes (Pathong et al., 1989; Murakami et al., 1993; Fahey and Stephenson, 2002; Tuchinda et al., 2002). In the most recent study, panduratin was shown to have antibacterial and antiviral activity (Rukayadi et al., 2010; Wu et al., 2011). Furthermore, 4-hydroxypanduratin A and panduratin A, isolated from the rhizomes of *B. rotunda* were found to show high inhibitory activity towards dengue-2 virus protease at 120 ppm (Kiat et al., 2006).

B. rotunda is traditionally propagated by vegetative techniques using rhizome segments which are protracted for large-scale multiplication. The lack of seed set in Zingiberaceae family makes conventional breeding methods inapplicable. Moreover, conventional propagation method allows transmission of soil borne

*Corresponding author. E-mail: ynorazma@yahoo.com Tel: +603 79674380. Fax: +603 79674178.

Abbreviations: BAP, N6-benzylaminopurine; Dicamba, 3, 6-dichloro-o-anisic acid; IAA, indole-3-acetic acid; MS, Murashige and Skoog (1962); NAA, α -naphthalene acetic acid; NE, non-embrogenic; 2, 4-D, 2, 4-dichlorophenoxy acetic acid.

pathogens and may spread diseases either to other plants or farming areas. Many of the Zingiberaceae species are susceptible to rhizome soft rot diseases, leaf spot and pathogens such as *Coleotrichum* species (Balachandran et al., 1990; Chan, 2004). In addition, *B. rotunda* consist only a small central rhizomes therefore producing limited buds to be used as propagules. Conversely, large size rhizome is the best source of planting material for plant growth and production. Conventional propagation requires appropriate maintenance and protection of seed rhizomes throughout the dormancy stage before next replanting process. Therefore, it is necessary to develop methods for *in vitro* propagation which is a simple and cost-effective way to obtain abundant uniform planting materials within a relatively short time (Çirak et al., 2007).

Callus cultures are tremendously important in plant biotechnology. It had been shown that shoot organogenesis *via* an intermediate callus phase can be used as an effective method for multiplication of other Zingiberaceae species (Malamug et al., 1991; Kackar et al., 1993; Salvi et al., 2001; Tan et al., 2005). However, no report has been published in plant regeneration of *B. rotunda* from morphogenic callus cultures, although reports on somatic embryogenesis and direct regeneration are available (Tan et al., 2005; Yusuf et al., 2011). This study will report on an efficient protocol for obtaining high yield of callus which is a potential starting material for clonal propagation.

MATERIALS AND METHODS

Establishment of aseptic explants

The mature healthy rhizome of *B. rotunda* collected from plants grown at the Botanical Garden, University of Malaya, were cleaned, rinsed and placed in an open container to allow shoots sprouting to 2 to 4 cm in length. The sprouting shoot buds were collected and washed under running tap water for at least 30 min, followed by soaking in 20% (v/v) commercial sodium hypochlorite (Clorox) for 15 min. After thorough washings in sterile distilled water, the shoot buds were surface sterilized with 0.1% (w/v) aqueous solution of mercuric chloride (HgCl₂) for 5 min. Following repeated washes with sterile distilled water, the meristem and shoot base explants (dissected into 2 mm cross section) were placed horizontally in 90 mm diameter Petri plates containing 25 ml medium.

Culture media and conditions

A culture medium containing Murashige and Skoog (1962) (MS) salts supplemented with 30 g/L sucrose and 2.0 g/L (w/v) agar was used in all experiments. The pH of the medium was adjusted to 5.7 by 1 N NaOH before being autoclaved at 121°C for 20 min. All cultures were incubated in the dark for callus induction and under a photoperiod of 16 h at 25 ± 2°C for shoot induction study.

Callus initiation

The shoot base explants cultured on MS medium complemented

with 1 mg/L α -naphthaleneacetic acid (NAA), 1.0 mg/L indole-3-acetic acid (IAA) and 1.0 mg/L α -biotin. Five different concentrations of 2, 4-dichlorophenoxy acetic acid (2, 4-D) (1.0, 2.0, 3.0, 5.0 and 10.0 mg/L) and 3, 6-dichloro-*o*-anisic acid (Dicamba) (0.2, 0.5, 0.8, 1.0 and 2.0 mg/L) were also added in the media either in combination or alone. MS medium lacking growth regulators served as a control experiment. The data on callus induction response was collected after 8 weeks of culture on callus induction medium.

To determine the best time for sub-culture, calli were removed from the original explants prior to culture on new media and the callus growth was monitored at weekly interval for 8 weeks by measuring the fresh weight (FW). Callus growth rate was observed and calculated using the equation described by Holme and Petersen (1996). After identifying the most suitable medium for callus proliferation, the calli were sub-cultured when they were at early stationary phase according to the callus growth curve. The cultures were observed periodically and morphological changes were recorded at regular intervals.

Shoot induction and multiplication

The calli growing on induction medium were transferred to the regeneration medium for 8 months and sub-cultured at 4 weeks interval. To study the effect of growth regulators on the regeneration of *B. rotunda* calli, regeneration medium was supplemented with either kinetin or 6-benzylaminopurine (BAP) at 5 different concentrations, 0.5, 1.0, 2.0, 5.0 and 10.0 mg/L individually. The cultures were kept under similar conditions used for callus induction studies. When the color of calli developed into opaque white, the cultures were kept under 16 h photoperiod to enhance further development. The regeneration capacity was evaluated after 8 weeks of culture.

Histological studies

Histological sections using resin were done to differentiate between embryogenic and non-embryogenic callus produced throughout this study. The samples were sliced according to the size of the available mold and then fixed for 24 to 48 h at room temperature in a glutaraldehyde-paraformaldehyde-caffeine (GPC) fixative solution (50.0 ml 0.2 M phosphate buffer, pH 7.2; 20.0 ml 10% (v/v) paraformaldehyde; 4.0 ml 25% (v/v) glutaraldehyde; 1.0 g caffeine and topped up to 100.0 ml with distilled water). The samples were then dehydrated in ascending ethanol concentration series (v/v): 30% (30 min); 50% (45 min); 70% (45 min); 80% (60 min); 90% (60 min); 95% (60 min), and twice in absolute ethanol for 60 min each. The tissues were then prepared for infiltration with basic resin (Leica Historesin Embedding Kit) for 24 to 48 h at 48°C under slight vacuum.

The sinking of a slightly translucent specimen underneath indicated the completion of infiltration. Then the specimens were embedded into molds and the resin was allowed to be fully polymerized; holders were attached and 3 μ m sections were sliced using a microtome.

Good sections were then stained with 1% periodic acid for 5 min, rinsed four times with distilled water at pH 4.5 and then submerged in Schiff's reagent (1.0 g basic fuchsin, 2.0 g disodium metabisulfite in 1 M HCl, 0.5 g neutralized activated charcoal) for 20 min in the dark. The slides were rinsed four (4) times with distilled water (pH 4.5). Finally, for counter staining, Naphthol Blue Black (1.0 g Naphthol Blue Black in 100 ml 7% acetic acid) was used at 60°C for 5 min. After further rinsing under running water, the sections were dried before viewing under a light microscope. All images were photographed using Leica Microsystems (EC3) (Switzerland).

Table 1. Effect of dicamba and 2,4-D on callus induction from shoot base explants of *B. rotunda* cultured on MS medium after four weeks ($n = 30$).

Auxin	Control					Dicamba					2,4-D				
Concentration (mg/L)	0.0	0.2	0.5	0.8	2.0	1.0	2.0	3.0	5.0	10.0					
Callogenesis response (%)	6.7	30.0	26.7	13.4	6.7	30.0	53.4	33.3	6.7	3.3					
Embryogenic callus (%)	0	0	0	0	0	13.3	36.7	16.7	0	0					
Non embryogenic callus (%)	6.7	30	26.7	13.4	6.7	16.7	16.7	16.7	6.7	3.3					

Rooting and acclimatization

The regenerated shoots measuring 4 to 5 cm in length were excised from callus clump and cultured on plant growth regulator (PGR) free MS medium to allow single plantlet development. Plantlets were then sub-cultured on MS medium supplemented with 2 g/L activated charcoal to promote rooting. Plantlets with well-developed shoots and roots were removed from the culture medium, washed gently under running tap water and transferred to plastic pots containing garden soil. Potted plantlets were covered with a transparent plastic bag to ensure high humidity and watered every day for two weeks. Transparent plastic bags were opened after two weeks prior to field transplantation. After four weeks, these plants were transferred to pots containing garden soil, sand (1:1 w/w) and maintained in a field under normal day length conditions.

Statistical analysis

Multiple range tests were conducted to determine the significant differences between mean samples.

RESULTS AND DISCUSSION

Shoot base explants cultured on MS medium supplemented with dicamba or 2,4-D showed callusing after 4 weeks of inoculation. Swelling of the explants was observed within 1 to 2 weeks, followed by emergence of callus after another 2 weeks. Callusing commenced at the cut surface of the explants and finally covered the whole surface of the explants completely. Incubation of explants on auxin-free MS medium (control) produced non-embryogenic (NE) calluses with 6.7% of callusing response. On the other hand, the response was higher in cultures complemented with auxin than control. The explants gave 6.7 to 30% callusing response on medium supplemented with different concentrations of dicamba. The response fraction decreased gradually from 30, 26.7, 13.4 and finally 6.7% as the dicamba concentration increased from 0.2, 0.5, 0.8 and 2.0 mg/L, respectively (Table 1). All cultures obtained from dicamba augmented medium produced NE callus as observed through morphological and histological studies. NE callus showed large, spongy, soft and wet structures (Figure 1A). Histological section illustrated that these cells were vacuolated and did not comprise of embryogenic cells (Figure 1B). The NE callus showed blue coloration when double-stained using established method of Gupta and Durzan (1987). These results showed that MS medium

supplemented with dicamba at different concentrations failed to provide favorable conditions for the production of embryogenic callus for *B. rotunda*.

Callus induction was facilitated in MS media supplemented with 2,4-D at all concentrations tested (Table 1). The cultures produced better response of callusing at lower concentration of 2,4-D compared to higher ones. The highest frequency (53.4%) of explants producing callus was achieved on medium containing 2.0 mg/L 2,4-D. Callus induced from media supplemented with low concentration of 2,4-D (1.0 to 3.0 mg/L) was observed to be embryogenic but contrary observation was made at higher concentrations (5.0 and 10.0 mg/L). The best response in terms of percentage of explants forming embryogenic calluses was on MS medium supplemented with 2.0 mg/l 2,4-D (36.7%). The percentage of response, growth and morphology of callus were influenced by the type of growth regulators used and their concentrations. Morphological observation of embryogenic callus under the stereo microscope showed yellow and friable structures (Figure 1C). The embryogenic callus revealed dense cytoplasm with prominent nuclei and laden with protein giving rise to blue-black stained cells (Figure 1D). This embryogenic callus characteristic was in accordance with the intense absorption of red acetocarmine when double-stained with 1% (w/v) acetocarmine and Evan's blue method (Tan et al., 2005). The differences in the percentages of cultures forming embryogenic calluses were negligible between 1.0 and 3.0 mg/L 2,4-D. Upon increasing the concentration of 2,4-D from 3.0 to 5.0 mg/L, a gradual decrease in percentage of explants forming callus was observed whilst 10.0 mg/L 2,4-D resulted in no callusing response. The outcome showed that 2,4-D was essential for *B. rotunda* to generate embryogenic callus.

From previous reports on Zingiberaceae species, the highest amount of callus formation was in 0.5 mg/L 2,4-D in combination with 1.0 mg/L BAP supplemented media for ginger meristem tips (Malamug et al., 1991). Dicamba and picloram (2.0 mg/L) or 5.0 mg/l NAA in combination with 0.5 mg/L BAP were used to produce callus from tumeric leaf base (Salvi et al., 2001). For ginger (*Zingiber officinale*), leaf derived callus was obtained at 58 and 52% using 1.0 and 1.5 mg/L 2,4-D, respectively, but the percentage reduced to 21% at 3.0 mg/L 2,4-D (Kackar et al., 1993). Similar results were reported in this study

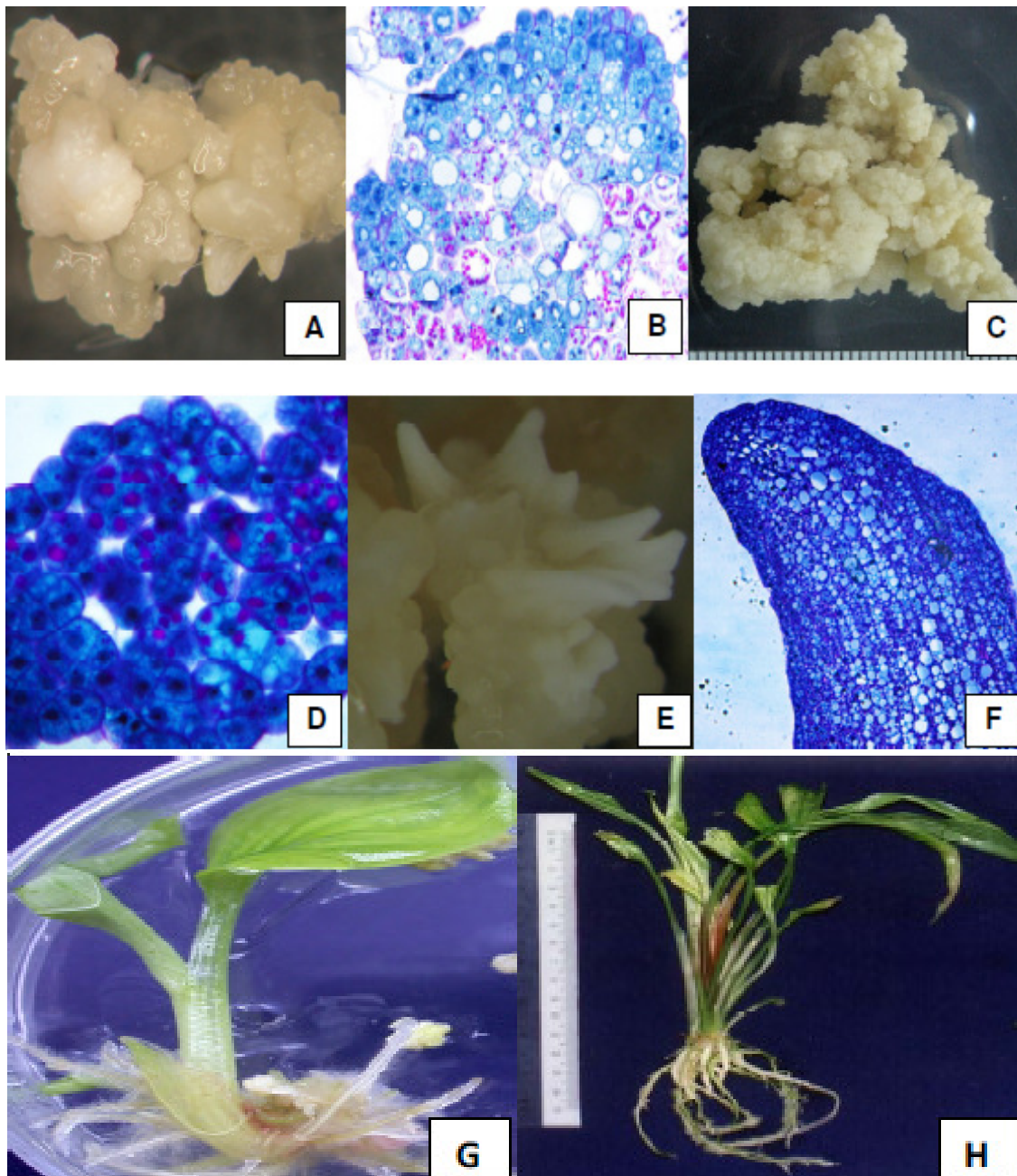


Figure 1. Induction of callus, types, morphology, histology and shoot regeneration of *B. rotunda*. (A) Morphology of NE callus, (B) histology of NE callus, (C) morphology of nodular callus, (D) histological section of nodular callus, (E) differentiation of callus into white primordial shoot, (F) histology of white primordial shoot, (G) shoot development on MS + 2 mg/L BAP, and (H) developed plantlet in MS medium supplemented with activated charcoal.

where a declined callus development was a function of higher concentration of 2,4-D.

For proliferation of callus, sub-culturing was carried out in MS medium supplemented with different concentrations of dicamba or 2,4-D (Table 2). Callus growth rate was calculated and found to be within similar range for all treatments, that is, 0.35 to 0.38 g per week. To obtain

high growth rate (g/week), the callus cultures could be grown in MS media supplemented with either Dicamba (0.5 to 0.8 mg/L) or 2,4-D (1 to 3 mg/L). The result showed that the concentration of these PGR significantly ($p < 0.5$) affected the growth rate of callus cultures. Based on these results, all further sub-culturing was carried out in MS medium supplemented with 3.0 mg/L

Table 2. Callus growth and regeneration rate on proliferation media.

PGR	Dicamba				2-4 D		
Concentrations (mg/L)	0.2	0.5	0.8	2.0	1.0	2.0	3.0
Growth rate (g/week, mean \pm S.D.)*	0.36 \pm 0.007	0.36 \pm 0.008	0.37 \pm 0.004	0.37 \pm 0.006	0.35 \pm 0.005	0.36 \pm 0.008	0.38 \pm 0.004
Regeneration rate (% , mean \pm S.D.)*	0	0	0	0	0	0	42 \pm 0.004

*Multiple range test was conducted for test of significance ($n = 10$).

2,4-D which showed maximum rate of proliferation (0.38 ± 0.004 g/week). Similar findings were reported by Tan et al. (2005), which showed that the fastest callus proliferation was observed in MS medium supplemented with 3.0 mg/L of 2,4-D. The growth rates were comparable for the second and third sub-cultures which subsequently decreased gradually (data not shown). Malamug et al. (1991) and Tan et al. (2005) showed similar pattern of growth proliferation of ginger and *B. rotunda*, respectively.

After identifying the most suitable medium for callus proliferation, growth curve of callus culture was determined. Biomass profile based on fresh weight in callus cultures revealed that the highest yield was obtained after the third week of culture. During the second and third week, calli were found to be in exponential phase. However, after the third week, the growth of the callus started to enter early stationary phase (Figure 2). The callus growth was negligible as the culture progressed into eighth week. After 3 months, the callus started to turn brown and eventually became necrotic possibly due to depletion of nutrients. Accumulation of toxic products and other limiting factors might have led to cells death and eventually a decline in biomass after several weeks of culture. Based on these results, the appropriate period for sub-culture was approximately on the fourth week of culture, which was between the end of exponential and early

stationary phases.

Plantlets were generated directly from the original calluses cultured on 3.0 mg/L 2,4-D ($42 \pm 0.004\%$) supplemented MS medium. These plantlets were produced only from nodular callus which showed the best morphology of callus to ensure further development and plant regeneration. The initial callus stage was crucial in tissue culture which determined a successful percentage of plantlet regeneration (Çirak et al., 2007). In order to improve regeneration capacity of calli, the embryogenic callus was sub-cultured on MS medium containing different concentrations of BAP or kinetin. The callus developed into somatic embryos during first sub-culture under a 16/8 h photoperiod. Subsequently, the callus turned to opaque white, and later became green forming organized structures (Figures 1E to F). Adventitious shoot buds were initially observed from the surface of callus within four weeks of culture and subsequently formed leaves. Shoot elongation took place after another month (Figure 1G). The eventual period from callus initiation to complete plant formation was within 2 months. The callus culture inoculated on MS medium responded differently to various concentrations of cytokinin (BAP and kinetin) (Table 3). The highest regeneration frequency ($49.8 \pm 0.5\%$) was achieved with 2.0 mg/L BAP supplemented MS medium. When the concentration of BAP was increased up to 5.0 mg/L, a decrease in

regeneration ability was observed. On MS medium supplemented with 0.5 to 10.0 mg/L kinetin, the highest regeneration frequency ($28.6 \pm 0.4\%$) was recorded at 1.0 mg/L kinetin.

Developments of somatic embryogenesis into complete plantlets have been reported previously for some Zingiberaceae species. Malamug et al. (1991) revealed that low concentrations (1.0 to 3.0 mg/L) of BAP resulted in successful shoot induction from ginger rhizome derived calli. The finding in this study agrees with that of Kackar et al. (1993), who described prominent regeneration at 2.0 mg/L BAP from ginger leaf derived callus. Nevertheless, higher level of BAP (5.0 mg/L) was used to induce green shoot primordia of turmeric callus while half strength MS medium supplemented with 1.0 mg/L kinetin was employed for further development of shoots (Salvi et al., 2001). Instead of MS medium supplemented with cytokinin, Tan et al. (2005) reported that the regeneration of *B. rotunda* on MS medium with 3.0 mg/L 2,4-D only was sufficient for regeneration process from callus culture. However from this study, callus regenerated in MS medium supplemented with cytokinin (2.0 mg/L BAP) gave higher rate of regeneration of complete plantlets of *B. rotunda* compared to those in MS medium supplemented with 2, 4-D only. Plant growth regulators, particularly cytokinins and auxins, play an extremely significant task in the process of callus induction and its proliferation. However,

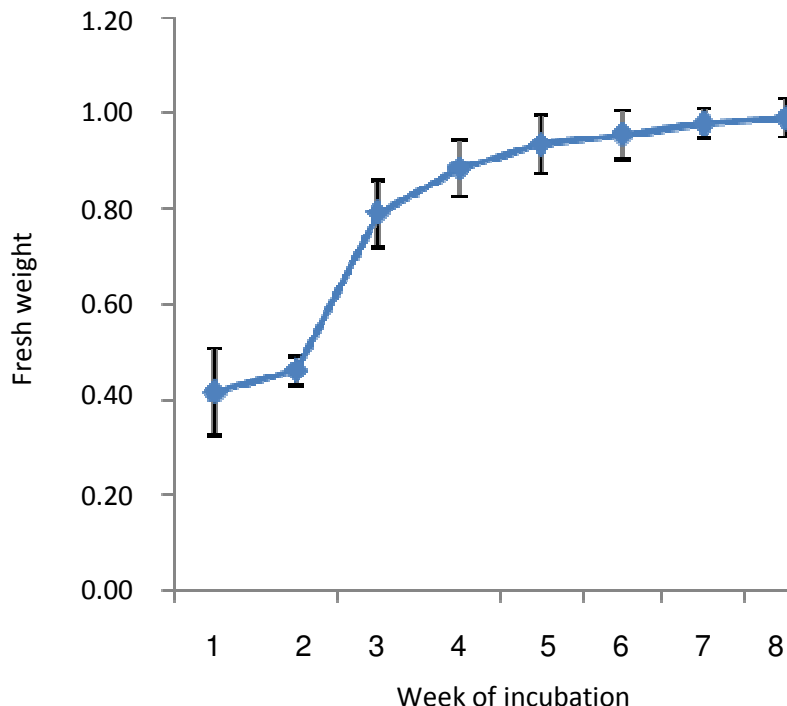


Figure 2. Growth curve of *B. rotunda* after 8 weeks of culture on MS medium supplemented with 3.0 mg/L 2,4-D.

Table 3. Effect of BAP and kinetin on shoot regeneration rate from shoot base segment of *B. rotunda* in MS medium after 8 weeks of culture.

Hormone concentration (mg/L)/type	0.5	1.0	2.0	5.0	10.0
BAP (% , mean \pm S.D.)*	31.2 \pm 0.5	45.6 \pm 0.4	49.8 \pm 0.5	10.2 \pm 0.1	0
Kinetin (% , mean \pm S.D.)*	15.5 \pm 0.2	28.6 \pm 0.4	22.8 \pm 0.3	26.7 \pm 0.4	0

*Multiple range test was conducted for test of significance ($n = 10$).

the optimum concentration for inducing callus differs according to the type of auxin and genotype (Varshney and Johnson, 2010). The present study revealed that different types and concentrations of cytokinin significantly affected the regeneration rate of *B. rotunda* callus cultures with the highest regeneration rate observed in MS medium supplemented with 2.0 mg/L BAP ($p < 0.05$).

In addition, in this study, the regeneration percentage reduced gradually upon sub-culture. According to previous work (Malamug et al., 1991; Tan et al., 2005), shoot-formation competency from ginger callus was reduced on the third sub-culture and regeneration which commenced during the first sub-culture produced better plantlets. Current investigation revealed that kinetin generally induced lower percentage of regeneration compared to BAP, with the highest percentage of plant regeneration at 49.8% (Table 3). Different responses of

cytokinins in this study, that is, BAP and kinetin was possibly due to dissimilarity in nutrient uptake, levels of endogenous growth regulators, and recognition by cells (Varshney and Johnson, 2010). Regeneration stage is highly reliant and controlled by growth regulators.

Meristem explants produced more abundant proliferating yellow nodular callus on MS medium containing 2.0 mg/L 2,4-D than shoot base explants (Table 4). The results in Table 4 indicated that these explants showed different callus growth response on similar media formulation. By culturing the meristem of *B. rotunda*, it could induce more callus formation with an average of 56.6% explants inducing embryogenic callus after eight weeks cultivation than shoot base explants. None of the meristem explants formed either non-embryogenic callus or organized structure such as roots and leaves. Whilst 30% of the shoot base explants produced callus and 26.7% of these explants formed

Table 4. Effect of explants on embryogenic callus formation of *B. rotunda* on MS medium supplemented with 1.0 mg/L NAA, IAA, *d*-Biotin and 2.0 mg/L 2,4-D (*n* = 30).

Explant	Embryogenic callus (%)	Non embryogenic callus (%)
Shoot base	30.0	26.7
Meristem	56.6	0.0

non-embryogenic callus. Some of the responses observed were either root or leaf formation from these explants. On the contrary, the meristem explants showed an increase in the number of explants forming embryogenic callus. Through observation, embryogenic callus derived from both explants remained normal with similar morphological characteristics. The present results showed the efficiency of the meristem explants in the induction of callus and concomitantly plant regeneration. This might be due to the presence of high auxins in young meristematic cells compared to mixture of young and mature cells in the shoot base explants.

To accomplish the establishment of plantlets in field conditions relied on the formation of vigorous root formation. In this study, plantlets were readily rooted and cultured individually on MS medium supplemented with activated charcoal (Figure 1H). The occurrence of root development on auxin-free medium was attributed to the likely presence of endogenous auxin in *in vitro* plantlets (Minocha, 1987). Hardening and acclimatization of *in vitro* plantlets were 100% successful. The *in vitro* raised plants did not show any observable phenotypic variation.

Conclusion

This study presented an efficient method for high yield production of callus masses of *B. rotunda* which is a valuable medicinal plant. The findings also pointed to the possibility of consistent mass production of reliable planting materials for large scale *in vitro* micropropagation.

ACKNOWLEDGEMENTS

We would like to thank the Malaysian Genomic Institute, Ministry of Science, Technology and Innovation, Public Service Department of Malaysia, University of Malaya and University Technology MARA (UiTM) for the financial support.

REFERENCES

Balachandran SM, Bhat SR, Chandel KPS (1990). *In vitro* clonal multiplication of turmeric (*Curcuma* spp.) and ginger (*Zingiber officinale* Rosc). Plant Cell Rep., 8: 521-524.
 Chan LK, Thong WH (2004). *In vitro* propagation of Zingiberaceae species with medicinal properties. J. Plant Biotechnol., 6: 181-188.

Cirak C, Ayan AK, Kevseroglu (2007). Direct and indirect regeneration of plants from internodal and leaf explants of *Hypericum bupleuroides* Gris. J. Plant Biol., 50: 24-28.
 Fahey JW, Stephenson KK (2002). Pinostrobin from honey and Thai ginger (*Boesenbergia pandurata*): A potent flavonoid inducer of mammalian phase 2 chemoprotective and antioxidant enzymes. J. Agric. Food Chem., 50: 7472-7476.
 Gupta PK, Durzan DJ (1987). Biotechnology of somatic polyembryogenesis and plantlet regeneration in loblolly pine. Biotechnol., 5:147-151.
 Holme IB, Petersen KK (1996). Callus induction and plant regeneration from different explant types of *Miscanthus x ogiformis* Honda 'Gingateus'. Plant Cell, Tissue and Organ Culture, 45: 43-52.
 Jaipetch T, Kanghae S, Pancharoen O, Patrick V, Reutrakul VA, Tuntiwachwuttikul P, White AH (1982). Constituents of *Boesenbergia pandurata*: Isolation, Crystal Structure and Synthesis of (±)-Boesenbergin A. Aust. J. Chem., 35: 351-361.
 Kackar A, Bhat SR, Chandel KPS, Malik SK (1993). Plant regeneration via somatic embryogenesis in ginger. Plant Cell, Tissue and Organ Culture. 32: 289-292.
 Kiat TS, Pippen R, Yusof R, Ibrahim H, Khalid N, Rahman NA (2006). Inhibitory activity of cyclohexenyl chalcone derivatives and flavonoids of fingerroot, *Boesenbergia rotunda* (L.), towards dengue-2 virus NS3 protease. Bioorganic and Medicinal Chemistry Letters, 16: 3337-3340.
 Larsen K (1996). A Preliminary checklist of the Zingiberaceae of Thailand. Thai For. Bull. (Bot.), 24: 35-49.
 Malamug JFF, Inden H, Asahira T (1991). Plantlet regeneration and propagation from ginger callus. Scient. Hort., 48: 89-97.
 Minocha SC (1987). Plant growth regulators and morphogenesis in cell and tissue culture of forest trees (1987). In: Bonga JM, Durzan DJ. (eds) Cell and Tissue Culture in Forestry. Martinus Nijhoff Publ., Dordrecht, pp. 50-66.
 Murakami A, Kondo A, Nakamura Y, Ohigashi H, Koshimizu K (1993). Possible anti-tumor promoting properties of edible plants from Thailand, and identification of an active constituent, cardamonin of *Boesenbergia*. Biosci. Biotech. Biochem., 57: 1971-1973.
 Murashige T, Skoog F (1962). A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol. Plant, 15: 473-497.
 Pandji C, Grimm C, Wray V, Witte L, Proksch P (1993). Insecticidal constituents from four species of the Zingiberaceae. Phytochem., 34: 415-419.
 Pathong A, Tassaneeyakul W, Kanjanapothi D, Tuntiwachwuttikul P, Reutrakul V (1989). Antiinflammatory activity of 5, 7-methoxyflavone. Planta Med., 55: 133-136.
 Rukayadi Y, Han S, Yong D. and Hwang JK (2010). In Vitro antibacterial activity of panduratin a against enterococci clinical isolates. Biol. Pharm. Bull., 33: 1489-1493.
 Salvi ND, George L, Eapen S (2001). Plant regeneration from leaf base callus of turmeric and random amplified polymorphic DNA analysis of regenerated plants. Plant Cell, Tissue Organ Cult., 66: 113-119.
 Tan SK, Pippen R, Yusof R, Ibrahim H, Rahman N, Khalid N (2005). Simple one-medium formulation regeneration of fingerroot [*Boesenbergia rotunda* (L.) Mansf. Kulturpfl. Via somatic embryogenesis. In Vitro Cell. Dev. Biol. Plant, 41: 757-761.
 Trakoontivakorn G, Nakahara K, Shinmoto H, Takenaka M, Onishi-Kameyama M, Ono H, Yoshida M, Nagata T, Tsushida T (2001). Structural analysis of a novel antimutagenic compound, 4-hydroxypanduratin A, and the antimutagenic activity of flavonoids in a Thai spice, fingerroot (*Boesenbergia pandurata* Schult.) against mutagenic heterocyclic amines. J. Agric. Food Chem., 49: 3046-50.

- Tucinda P, Reutrakul V, Claeson P, Pongprayoon U, Sematong T, Santisuk T, Taylor WC (2002). Anti-inflammatory cyclohexenyl chalcone derivatives in *Boesenbergia pandurata*. *Phytochem.*, 59: 169-173.
- Varshney A, Johnson TS (2010). Efficient plant regeneration from immature embryo cultures of *Jatropha curcas*, a biodiesel plant. *Plant Biotechnol.Reports*, 4: 139-148.
- Wu N, Kong Y, Zu Y, Fu Y, Liu Z, Meng R, Liu X, Efferth T (2011). Activity investigation of pinostrobin towards herpes simplex virus-1 as determined by atomic force microscopy. *Phytomedicine*, 18: 110-118.
- Yusuf NA, Annuar MSM, Khalid N (2011). Rapid micropropagation of *Boesenbergia rotunda* (L.) Mansf. Kulturpfl. (a valuable medicinal plant) from shoot bud explants. *Afr. J. Biotechnol.*, 10: 1194-1199.