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A Medicinal Ginger, *Boesenbergia rotunda*: From Cell Suspension Cultures to Protoplast Derived Callus

(Halia perubatan, *Boesenbergia rotunda*: Daripada Kultur Sel Penggantungan kepada Kalus daripada Protoplas)

HAO-CHEAK TAN, BOON-CHIN TAN, SHER-MING WONG & NORZULAANI KHALID*

ABSTRACT

Boesenbergia rotunda *is a medicinal ginger that has been found to contain several bioactive compounds such as boesenbergin A, panduratin A, cardamonin, pinostrobin and pinocembrin. These compounds are useful in treating various ailments, such as oral diseases, inflammation and have also been used as an aphrodisiac. In this study, an efficient protocol for developing and isolating protoplast cultures for* B. rotunda *has been established. Rhizome buds of* B. rotunda *were used as explants to initiate callus growth and the established cell suspension cultures were used to optimize their growth conditions. Our results indicated that embryogenic suspension cultures in liquid Murashige and Skoog (MS) medium supplemented with 3% (w/v) sucrose produced the highest growth rate (μ = 0.1125), whereas no promotive effect was seen in the presence of 2,4-dichlorophenoxyacetic acid and those that underwent sonication treatment. Amount of protoplasts isolated ranging from 1-5 × 10^5 protoplast per mL were isolated using 0.25% (w/v) macerozyme and 1% (w/v) cellulase for 24 h under continuous agitation (50 rpm) in dark condition. Of the isolated protoplasts, 54.93% were viable according to fluorescein diacetate staining test. Micro-colonies were recovered in liquid MS medium containing 9 g/L mannitol, 2 mg/L 1-naphthaleneacetic acid and 0.5 mg/L benzylaminopurine (BAP) for 4 weeks and subsequently transferred to solid MS medium supplemented with 0.5 mg/L BAP for callus initiation. The protoplast system established in this study would be useful for genetic manipulation and modern breeding program of* B. rotunda.

Keywords: Cell suspension culture; medicinal ginger; micropropagation; protoplasts

ABSTRAK

Boesenbergia rotunda *ialah halia ubatan yang didapati mengandungi beberapa sebatian bioaktif seperti boesenbergi A, panduratin A, cardamonin, pinostrobin dan pinocembrin. Sebatian ini berguna dalam merawat pelbagai penyakit seperti penyakit mulut, keradangan dan juga telah digunakan sebagai afrodisiak. Dalam kajian ini, satu protokol berkesan untuk membangun dan mengasingkan budaya protoplas untuk* B. rotunda *telah dibentuk. Tunas rizom* B. rotunda *telah digunakan sebagai eksplan untuk memulakan pertumbuhan kalus dan kultur penggantungan sel yang telah dibentuk digunakan untuk mengoptimumkan keadaan pertumbuhan mereka. Hasil kajian kami menunjukkan bahawa kultur penggantungan embriogenik dalam medium cecair Murashige dan Skoog (MS) ditambah dengan 3% (w/v) sukrosa menghasilkan kadar pertumbuhan yang paling tinggi (μ = 0.1125), manakala tiada kesan penggalakan dilihat dengan kehadiran asid 2,4-dichlorophenoxyacetic dan orang-orang yang menjalani rawatan sonikasi. Jumlah protoplas yang diasingkan adalah antara 1-5 × 10^5 setiap mL telah diasingkan menggunakan 0.25% (w/v) maserozim dan 1% (w/v) selulase untuk 24 h bawah penggoncangan berterusan (50 rpm) dalam keadaan gelap. Daripada pencilan protoplas, 54.93% adalah berdaya maju mengikut ujian pewarnaan fluoresein diasetat. Micro-koloni ditemui dalam medium cecair MS yang mengandungi 9 g/L manitol, 2 mg/L 1-naftalenaasetik asid dan 0.5 mg/L benzylaminopurine (BAP) selama 4 minggu dan kemudiannya dipindahkan kepada medium pepejal MS ditambah dengan 0.5 mg/L BAP untuk permulaan kalus. Sistem protoplas yang dibentuk dalam kajian ini akan berguna untuk manipulasi genetik dan program pembiakan moden* B. rotunda.

Kata kunci: Halia ubatan; kultur penggantungan sel; mikrorambatan; protoplas

INTRODUCTION

Protoplast is a plant cell that has its cell wall completely or partially removed either enzymatically or mechanically (Jiang et al. 2013). Under appropriate chemical and physical stimuli, each protoplast has the potential to regenerate a new cell wall and undergo repeated mitotic division to produce daughter cells that can be regenerated into plantlets (Davey et al. 2005). Protoplast is a useful biological system that has been widely used to study cell fusion, somaclonal variation, genetic transformation and plant breeding on various plant species (Aoyagi 2011; Yeong et al. 2008). It has allowed tremendous progress in understanding the event of cell wall formation, cell division and proliferation (Pati et al. 2005). Besides,

metabolite transport between different intracellular compartments has been studied by isolating individual organelles from the protoplasts (Park et al. 2012). Besides allowing good visual images at organelle and cellular levels when stained, protoplasts also serve as a good candidate for high throughput screening of cells with high expression during transformation (Kirchhoff et al. 2012). This has made protoplasts to be commonly used as a gene expression system rather than as a platform technology for gene manipulation. Only low level of chimerism was observed in transformed individuals using this technique (Pindel 2007). With the advancement of high throughput selection methods, protoplasts can be easily selected and subsequently induced to form callus and whole plants. Protoplast isolation has now become a routine for a wide range of species, such as banana (Khatri et al. 2010), cucumber (Huang et al. 2013) and guava (Rezazadeh & Niedz 2015).

Despite the significant progress made in establishing protoplast culture, several important factors must be considered carefully to ensure high success rate. These include the source of tissues, composition of cell wall, types of enzymes used, incubation period, pH, speed of agitation as well as osmotic pressure (Zhou et al. 2008). For instance, different protoplast sources, such as hypocotyls, leaves (Grzebelus et al. 2011) and embryogenic calli (Jumin 2013), require different enzymes to isolate protoplasts as they have different intra- and intercellular tissues compositions (Ratanasanobon & Seaton 2013). Besides, the ability of protoplasts and protoplast-derived cells to develop into fertile plants is greatly influenced mostly by same factors, such as the source of tissue as well as culture medium and environmental factors (Davey et al. 2005). Many efforts have been made in refining the methodologies for protoplast isolation and maintenance. However, only a few successes have been reported in establishing protoplast cultures in medicinal ginger.

Boesenbergia rotunda is well-known for its medicinal properties and economical value. Its ethnomedicinal usage has drawn the attention of scientists to further investigate its medicinal properties. Several bioactive compounds have been successfully identified from the rhizome extract of *B. rotunda*, such as panduratin A, pinocembrin and 4-hydroxypanduratin. These compounds have been reported to exhibit anti-oxidant, anti-bacterial, anti-fungal, anti-inflammatory, anti-tumour, and anti-tuberculosis activities (Tan et al. 2015, 2012a). Despite the potential of these compounds, the limited continuous supply of plant source continues to be a significant challenge (Patel & Krishnamurthy 2013). *B. rotunda* is propagated by vegetative method. Nevertheless, this method is slow and time-consuming (Tan et al. 2015). The establishment of protoplasts provides a useful system to enable genetic manipulation in *B. rotunda* and to improve the yield of these useful bioactive compounds. Therefore, the aims of the present study were to establish an efficient protoplast isolation protocol and to optimize the growth conditions for *B. rotunda* suspension cultures.

Materials and Methods

Plant materials and maintenance of cultures

B. rotunda callus cultures were induced from rhizome buds according to Tan et al. (2005). Briefly, the explants were cultured on solid Murashige and Skoog (MS) (1962) medium supplemented with 1 mg/L D-biotin, 1 mg/L indole-3-acetic acid, 2 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), 1 mg/L 1-naphthylacetic acid (NAA), 30 g/L sucrose and 2 g/L gelrite. The suspension cultures were subsequently established and maintained according to Tan et al. (2012b) in liquid MS medium supplemented with 150 mg/L malt extract, 5 g/L maltose, 100 mg/L glutamine, 1 mg/L biotin, 1 mg/L 6-benzylaminopurine (BAP), 1 mg/L NAA, 2 mg/L 2,4-D and 30 g/L sucrose.

Optimization of the growth of cell suspension culture

To optimize the conditions of cell growth, cell suspensions were inoculated in liquid MS medium supplemented with different concentrations of 2,4-D (0, 2, 4, 8 and 16 mg/mL) and sucrose (0, 1.5, 3, 4.5 and 6% (w/v)). In order to determine the effect of sonication on cell growth, cell suspensions were sonicated at different times (0, 0.5, 2, 5 and 10 min) in a water bath sonicator. Settled cell volume (SCV) was measured at 3-day intervals until 27 days and specific growth rates (μ) of each treatment were calculated by using this formula: $\mu = (\ln (\text{Final Initial}^{-1}))\ \text{Time}^{-1}$. All cultures were incubated at 25 ± 2°C under a 16 h light and 8 h dark photoperiod with a light intensity of 1725 lux provided by cool white fluorescent light.

Isolation of protoplast

Ten mL of suspension culture containing 20% (v/v) settled cells were incubated with an equal volume of filter sterile enzymes in different concentrations and combinations (cellulase: 1 and 2% and macerozyme: 0.25 and 0.5%). The mixture was then incubated at 25 ± 2°C for 5, 24 and 48 h, respectively, under continuous agitation condition of 50 rpm. The mixture was filtered through a 80-μm nylon filter to separate protoplasts from the 'debris'. The filtrate was then centrifuged for 5 min at 80 × g. The sediment was washed and soaked with protoplast washing medium (CPW13M) consisted of 27.2 mg/L KH_2PO_4, 101 mg/L KNO_3, 1480 mg/L $CaCl_2.2H_2O$, 246 mg/L $MgSO_4.7H_2O$, 0.16 mg/L KI, 0.025 mg/L $CuSO_4.5H_2O$ and 130 g/L mannitol and floated on 8 mL of protoplast floatation medium (CPW21S) consisted of 27.2 mg/L KH_2PO_4, 101 mg/L KNO_3, 1480 mg/L $CaCl_2.2H_2O$, 246 mg/L $MgSO_4.7H_2O$, 0.16 mg/L KI, 0.025 mg/L $CuSO_4.5H_2O$ and 210 g/L sucrose without mixing. The 2-layer solution was then centrifuged at 120 × g for 10 min to allow the formation of protoplast ring layer. This layer was then transferred to 3 mL CPW13M for the maintenance of protoplasts shape and subsequent protoplast counting. The number of protoplast formed was counted using a

Fuchs-Rosenthal haemocytometer counting chamber. Protoplast density was adjusted to 1-5 × 10^5 protoplasts per mL using CPW13M and cultured with 5 mL liquid MS medium supplemented with 150 mg/L malt extract, 5 g/L maltose, 0.5 mg/L BAP, 2 mg/L NAA, 30 g/L sucrose and 90 g/L mannitol (MSP1 9M) in dark condition. The concentration of mannitol was adjusted from 9 to 5% (w/v) followed by 1% (w/v) using the same medium without mannitol supplementation (MSP1) in one week interval. Micro-colonies formed from the protoplasts were plated on solid MS medium containing 0.5 mg/L BAP and 0.2% (w/v) gelrite for callus induction.

PROTOPLAST STAINING

Protoplasts were stained with 70 µg/µL calcofluor white M2R (Fluorescent Brightener 28, Sigma, USA). After 10 min of incubation, the protoplasts were examined under an ultraviolet (UV) fluorescence microscope (Axiovert 10, Zeiss, Germany) emission at 488 nm. Protoplast viability (the percentage of protoplasts surviving the isolation and purification procedure) was determined using 100 µg/µL Fluorescein Diacetate (FDA) stain (Sigma, USA). The mixture was incubated for 15 min and then examined under a UV fluorescence microscope.

STATISTICAL ANALYSIS

Data were analysed statistically by using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test at a significance level of $p<0.05$ using SPSS version 16.

RESULTS AND DISCUSSION

EFFECTS OF 2,4-D, SUCROSE AND SONICATION ON CELL GROWTH

In this study, the growth of cell suspension cultures was influenced by sucrose but not growth regulator treatments. The highest growth rate (µ = 0.12/day) was observed in cell suspension cultured in MS medium supplemented with 1.5% (w/v) sucrose (Table 1). Whereas, cell suspension cultured in MS medium supplemented with 3% (w/v) sucrose showed the highest final SCV (µ= 0.11/day) at day 27 compared to the lower or higher concentrations of sucrose (Table 1). Cell suspension cultures in MS media containing 0% (w/v) and 1.5% (w/v) sucrose did not show any continuous growth after 18 days of culture. This finding indicated that low concentration of sucrose has negatively affected the cell growth as the plant cell cultures rely on simple carbon sources, such as sucrose, glucose and fructose for continuous growth and development (Rao & Ravishankar 2002). Sucrose has been considered to be one of the most effective carbon sources to improve plant growth. Abdullah et al. (1998) reported that culture media containing 3 to 5% (w/v) sucrose were able to improve the cell growth of *Morinda elliptica* suspension culture. Nevertheless, high concentration (more than 5% (w/v)) of sucrose might affect the water content in suspension cells due to osmotic pressure (Ho et al. 2010; Lee et al. 2006). High osmotic pressure has been reported to inhibit the nutrients uptake (Lee et al. 2006) and halted the cell cycle of suspension cells (Wu et al. 2006). Similar observation has been reported in *Holarrhena antidysenterica* (Panda et al. 1992) and *Panax notoginseng* (Zhang et al. 1996), where 4% and 6% sucrose, respectively, has been shown to decrease the cell growth.

Supplementation of 2,4-D in the MS media did not accelerate cell growth, whereas 2,4-D-free MS medium (days 6 to 18) produced the highest growth rate (µ= 0.07) compared to other treatments (Table 1). This might be due to the presence of 2,4-D that causes phytotoxicity effect in the suspension culture and render the cell growth (Tewes et al. 1984). 2,4-D has been considered as a specific limiting factor. Their presence within or outside the cells (between 4 × 10^{-8} to 4 × 10^{-6} M) has been reported to promote cell division, whereas higher concentration (>4 × 10^{-6} M) of 2,4-D might cease the cell division (Leguay & Guern 1975). Previous study reported poor cell growth and occurrence of plasmolysis when *Lycopersicon esculentum* suspension cultures inoculated in MS medium containing 2 mg/L 2,4-D (Tewes et al. 1984). Although 2,4-D is widely used for callus induction, however, it exhibits greater inhibitory effect to long-term compared to short-term suspension cultures. For instance, Patil et al. (2003) reported that long-term suspension cultures of *Lycopersicon chilense* in the medium containing 2,4-D have lost its vigour and high frequency of browning was recorded.

In order to determine the effect of sonication on suspension cultures, cells were sonicated for 0, 0.5, 2, 5

TABLE 1. Effect of different treatments on the specific growth rate of *Boesenbergia rotunda* suspension culture

Treatments	Specific growth rates (µ/d)
2,4-D (mg/L)	
0	0.07 ± 0.00 ^{a}
2	0.03 ± 0.01 ^{b}
4	0.04 ± 0.03 ^{ab}
8	0.04 ± 0.01 ^{ab}
16	0.03 ± 0.03 ^{b}
Sonication (s)	
0	0.03 ± 0.01 ^{s}
30	-0.01 ± 0.01 ^{tu}
120	-0.03 ± 0.01 ^{t}
300	-0.01 ± 0.00 ^{u}
600	-0.02 ± 0.02 ^{tu}
Sucrose (g/L)	
0	0.06 ± 0.00 ^{w}
15	0.12 ± 0.01 ^{x}
30	0.11 ± 0.00 ^{x}
45	0.10 ± 0.00 ^{y}
60	0.09 ± 0.01 ^{z}

Means indicated with the same letter were not significantly different based on analysis of variance (ANOVA) followed by Duncan's multiple-range test at $p < 0.05$

and 10 min. All sonicated suspension cultures exhibited negative growth rate, whereas the suspension cultures without sonication showed positive growth (Table 1). The ultrasound-induced fluid motion and hydrodynamic events generated from the sonicator might induce mechanical stress to the cells and affect the cell viability (Miller et al. 1996). Similar findings were reported by Bohm et al. (2000), where the viability of *Petunia hybrida* suspension culture was decreased from 95% to 35% under standing-wave condition. Their study indicated that the wave field created by a sonicator is able to reduce the cellular viability. A significant drop in the viability of the *Panax ginseng* suspension culture was observed a day after being exposed to ultrasound treatment, however, it gradually recovered after 2 to 3 days with a higher ultrasound power and longer exposure period (Wu & Lin 2002). Besides the exposure time, we suggest to optimize a few parameters in the future, such as acoustic energy density, mechanical properties and age of the cells, for maximizing the cell growth.

SOURCE OF PROTOPLASTS

In this study, 5-day old suspension cultures in the early logarithm phase were used as a source to isolate protoplasts (Figure 1). Suspension cultures in this phase consist of small cells with a thin cell wall which are suitable for protoplast isolation (Grosser & Gmitter Jr 2011). The cells were small and most probably thin cell-walled which can be easily digested. After early logarithm phase, suspension cells enlarged with a few large vacuoles and thicker cell wall negatively affected the yield of protoplast. On the other hand, isolation of protoplast from cell suspension cultures at the stationary phase remains technically challenge and may need a complex enzyme digestion as the cells start to lignify their cell wall at this stage (Schenk & Hildebrandt 1969).

ISOLATION OF PROTOPLASTS

Macerozyme at 0.25 and 0.5% (w/v) and cellulase at 1 and 2% (w/v) were used to isolate protoplasts. The highest protoplast yield was recorded when 2.0% (w/v) cellulase and 0.5% (w/v) macerozyme were used (Figure 2). Similar result was observed when the same ratio (4:1) of cellulase and macerozyme at 1.0 and 0.25% (w/v) was applied. Our results suggested that the ratio of cellulase and macerozyme enzymes were important in order to obtain good protoplasts yield. This was in agreement with the study carried out by Uchimiya and Murashige (1974), where the highest protoplasts yield observed in tobacco cells were isolated using both cellulase and macerozyme at the ratio of 5 to 1 instead of single enzyme. This might be due to the enzyme substrate specificity (Chen et al. 1994). Macerozyme (0.1 to 1.0% (w/v)) has been commonly used to isolate single cell from cell clumps or explants, while cellulase (0.5 to 5% (w/v)) has been used to digest cellular cell wall of the isolated cells (Fitzsimons & Weyers 1985).

Enzyme incubation time is also another critical factor to ensure high protoplast yield (Zhang et al. 2011). It might vary between plant species and their cell wall composition (Tee et al. 2010). In this study, three different enzyme incubation times (5, 24 and 48 h) with suspension cells were used. The results showed that enzymes incubated at 24 h produced the highest protoplast yield (1.96×10^5) (Figure 3). However, longer incubation time (48 h) did not increase the protoplast yield (Mazarei et al. 2011). Geetha et al. (2000) reported that 24 h was optimum to maximize the yield of protoplasts in cardamom suspension culture. In contrast, Reusink and Thimann (1965) reported that the protoplast yields for *Avena sativa* cell suspension cultures did not show any increment after 1 h of incubation. In addition, prolonged enzyme incubation time or over-digestion might cause the protoplasts to break and dysfunction (Zhang et al. 2011).

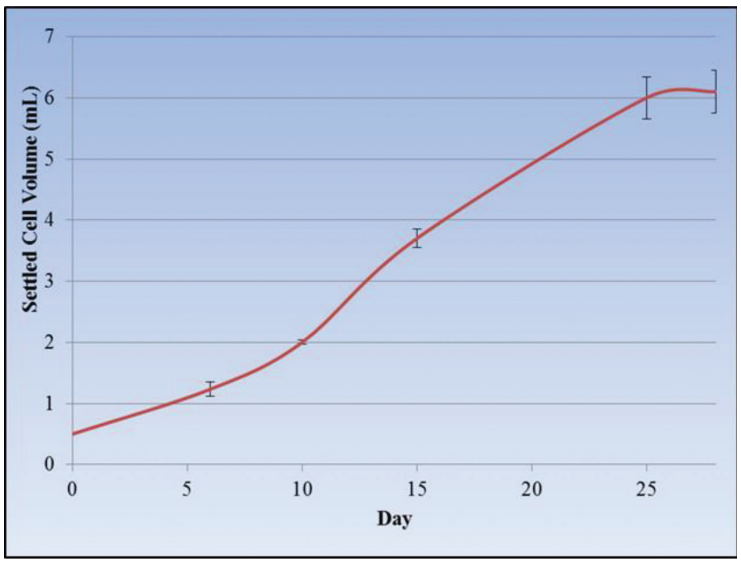

FIGURE 1. Standard growth curve for *Boesenbergia rotunda* cell suspension culture

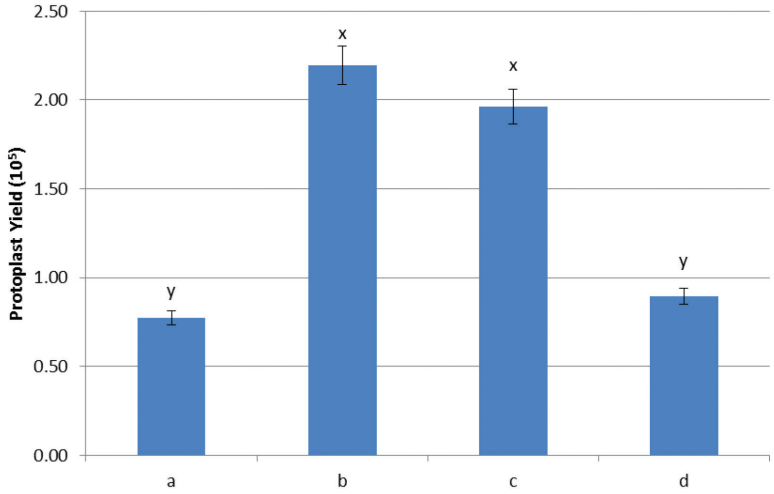

FIGURE 2. Effects of different combinations of enzymes on protoplast yield (a) 1% cellulase and 0.5% macerozyme (b) 2% cellulase and 0.5% macerozyme (c) 1% cellulase and 0.25% macerozyme (d) 2% cellulase and 0.25% macerozyme. Different letters indicate significant differences at $p < 0.05$ level; % indicates percentage of weight in volume

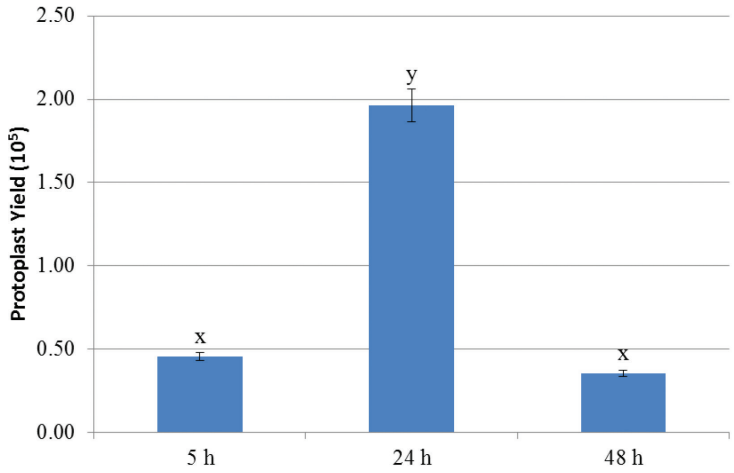

FIGURE 3. Effects of different incubation periods on protoplast yield. Different letters indicate significant differences at $p < 0.05$ level

PROTOPLASTS VIABILITY AND RECOVERY

Isolated protoplasts were spherical in shape and occurred as single cells after cell wall digestion (Figure 4(a)). Of the isolated protoplasts, 54.93% were viable according to FDA staining test (Figure 4(b) & 4(c)). The fluorescence resulted from intracellular hydrolysis of FDA that passed through cell membranes and accumulated inside the cell. The cell exhibited green fluorescence when excited by blue light. Protoplast recovery was carried out in liquid MS medium for about 4 weeks. Micro-colonies formed were then transferred to solid MS medium. The protoplasts started to develop to a 2-cell stage (Figure 5(a)) for the first five days, while 4-cell stage was observed at day 7 (Figure 5(b)). The formation of cell wall was confirmed by calcofluor white M2R staining. White fluorescence was observed (Figure 5(c) & 5(d)) in viable protoplasts with cell wall formation. Approximately 0.05% callus was formed with an initial plating density of 2×10^5 protoplasts per mL after 5 weeks of culture on solid MS medium supplemented with 0.5 mg/L BAP (Figure 5(e)).

Recovery of protoplasts is highly dependent on the plating density as it might affect 'cell-to-cell' communication between protoplasts (Ochatt & Power 1992). Inappropriate plating density hindered cell division in the protoplast culture due to nutrition depletion or lack of growth stimulus factors (Al-Khayri 2012). Previous study showed that the plating density within 0.5–10 × 10^5/mL was effective to recover protoplasts in many plant species (Davey et al. 2005). Besides, protoplasts can also be recovered using solid culture, bead culture, nurse culture and nurse culture with a feeder layer (Smith et al. 1984). In summary, an efficient protocol for isolating protoplasts

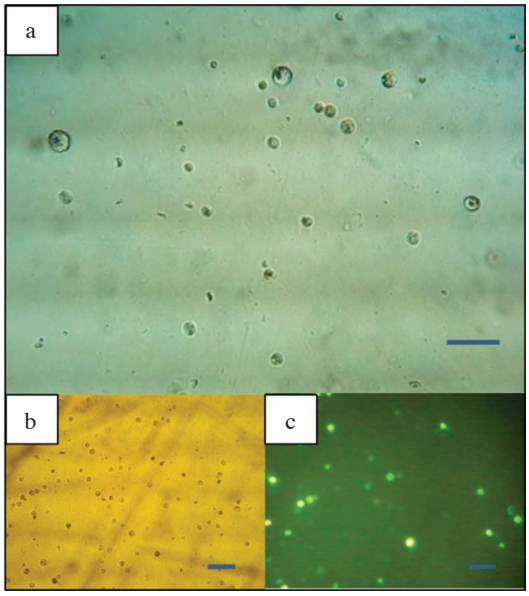

FIGURE 4. Protoplasts isolated from *Boesenbergia rotunda* cell suspension culture under observation using an inverted microscope (a) isolated protoplast, bar = 125 µm (b) protoplasts stained with FDA viewed under normal light, bar = 125 µm (c) viable protoplasts appeared green fluorescence under UV light, bar = 125 µm

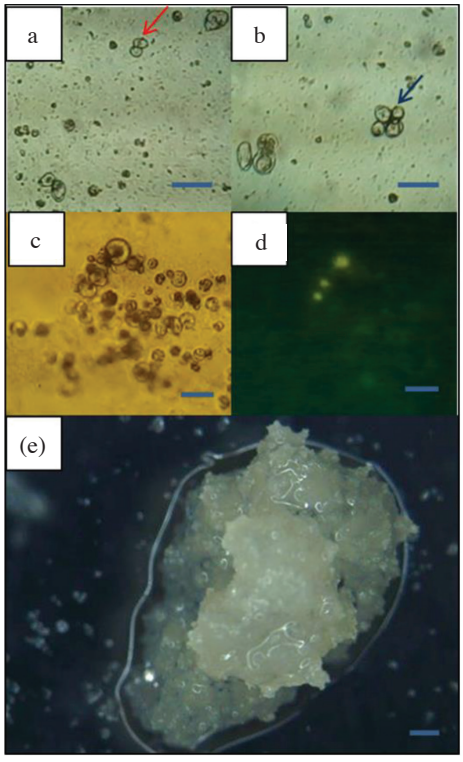

FIGURE 5. Recovery of the protoplasts at different developmental stages (a) 2-cell stage for first 5 days, red arrow indicates 2-cell stage, bar = 100 µm (b) 4-cell stage at day 7, blue arrow indicates 4-cell stage, bar = 100 µm (c) protoplasts stained with calcofluor white M2R after 24 h of culturing viewed under normal light, bar = 500 µm (d) cell wall appeared white fluorescent under UV light, bar = 500 µm (e) friable callus derived from protoplast, bar = 1 mm

from *B. rotunda* has been established. This study represents a foundation for further research which could be applied in crop improvement program using protoplasts fusion and genetic transformation technologies. To the best of our knowledge, protoplast technology in *B. rotunda* cell suspension culture has not been reported so far.

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Hao-Cheak Tan, Sher-Ming Wong & Norzulaani Khalid*
Institute of Biological Sciences
Faculty of Science, University of Malaya
Lembah Pantai, 50603 Kuala Lumpur
Malaysia

Boon-Chin Tan & Norzulaani Khalid*
Centre for Research in Biotechnology for Agriculture (CEBAR)
University of Malaya
Lembah Pantai, 50603 Kuala Lumpur
Malaysia

*Corresponding author; email: lani@um.edu.my

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