1.0 Introduction

Boesenbergia rotunda is a perennial monocotyledonous ginger that belongs to the Zingiberaceae family. It is a small herbaceous plant with short and fleshy rhizomes that possess aromatic characteristics and a slight pungent taste (Sudwan *et al*., 2007). Traditionally, it is used as food spices (Chan *et al*., 2008) and folk medicines to treat stomach ache, women discomforts and after birth confinement (Ching *et al*., 2007; Morikawa *et al*., 2008). In recent years, 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 A (Tan *et al*., 2012a; 2012b). These compounds have been reported to exhibit antioxidant, antibacterial, antifungal, anti-inflammatory, antitumour and anti-tuberculosis activities (Tan *et al*., 2012a; 2012b).

B. rotunda is traditionally propagated by vegetative techniques using a rhizome segment (Yusuf *et al*., 2011a). Low proliferation rate, soil-borne disease infection and degeneration of rhizomes continue to be significant limitations in ginger propagation (Guo *et al*., 2007). Studies on the micropropagation of *B. rotunda* have been reported using shoot bud and shoot-derived callus cultures for rapid and large scale production (Yusuf *et al*., 2011a; 2011b). However, limited tissue culture system is amendable for genetic improvement and variant development. Therefore, an alternative approach lies on employing protoplast technique to develop elite or disease resistant varieties for *B. rotunda*.

Protoplast has been widely used to study somaclonal variation, genetic transformation and plant breeding program on various plant species, including rice (Chen *et al*., 2006), tobacco (Rehman *et al*., 2008), maize and *Arabidopsis* (Sheen, 2001). However, establishment and regeneration of protoplasts remain technically challenging. Several factors usually influence the protoplast yield, such as the source of tissues, composition of cell wall, types of enzymes used, incubation period and pH, speed of agitation as well as osmotic pressure (Davey *et al*., 2005; Zhou *et al*., 2008). Therefore, the aims of the present study were to optimise the conditions for maintaining *B. rotunda* suspension cultures and to establish an efficient protoplast isolation protocol. To our knowledge, protoplast technology in *B. rotunda* cell suspension culture has not been reported so far.

The objectives of this study were:

1. To maximise the growth of suspension cultures in order to obtain good protoplast yield that could undergo cell division
2. To optimise protoplasts isolation protocol using different enzyme combinations and incubation times
3. To recover viable protoplasts that eventually formed callus

2.0 Literature review

2.1 Classification

Zingiberaceae belongs to the family of ginger which consists of about 1200 species distributed throughout tropical Asia. 1000 species are found abundantly in South East Asia, such as Malaysia, Indonesia, Brunei, Singapore, the Philippines and Papua New Guinea. The Zingiberaceae family consists of 2 subfamilies (Costoideae and Zingiberoideae). The Costoideae consists of 1 tribe (Costeae) with only 1 genus (Costus), while the Zingiberoideae is sub-divided into 3 tribes (Globeae, Hedychieae and Alpiniae). The tribe Globbeae has only 1 genus (Globba). There are 8 genera under the tribe Hedychieae, namely Zingiber, Curcuma, Hedychium, Comptandra, Scaphochlamys, Boesenbergia, Kaempferia and Haniffia. The tribe Alpinieae consists of 13 genera, where the most common genera are Alpinia, Phaeomeria, Achasma, Amomum and Elettaria.

2.2 *Boesenbergia rotunda*

2.2.1 Morphological description

B. rotunda [formerly known as *Kaempferia pandurata* Roxb. or *Boesenbergia pandurata* (Roxb. Schltr)] belongs to the Zingiberaceae family. It is a perennial monocotyledonous herb which is also known as chinese key, finger root or "temu kunci". Among the species, *B. rotunda* is the most abundant species found in Malaysia (Bhamarapravati *et al.*, 2006; Ching *et al.*, 2007). It is a small herbaceous plant with short, fleshy rhizomes that possess aromatic characteristics and a slightly pungent taste (Tuchinda *et al.*, 2002; Sudwan *et al.*, 2007) (Figure 2.1).

2.2.2 Common uses

The rhizome of *B. rotunda* is well-known for its medicinal and economical significance (Figure 2.2). It mostly used as food spices (Chan *et al.*, 2008) and traditional medicines against inflammation, aphthous ulcer, dry mouth, stomach discomfort, dysentery, leucorrhoea, oral diseases, cancers, and kidney disorders (Morikawa *et al.*, 2008). Besides, rhizomes have also been considered as an effective tonic for women after giving birth and serve as a remedy in postpartum protective medication and treatment for rheumatism (Chomchalow *et al*., 2006; Ching *et al*., 2007; Sudwan *et al*., 2007).

Figure 2.1: Plant of *B. rotunda*. **A:** whole plant with maroon stem. **B:** shoots with 3 to 5 leaves attached to maroon sheaths. **C:** leaf with 7 to 9 cm broad and 10 to 20 cm long (Yusuf, 2011c).

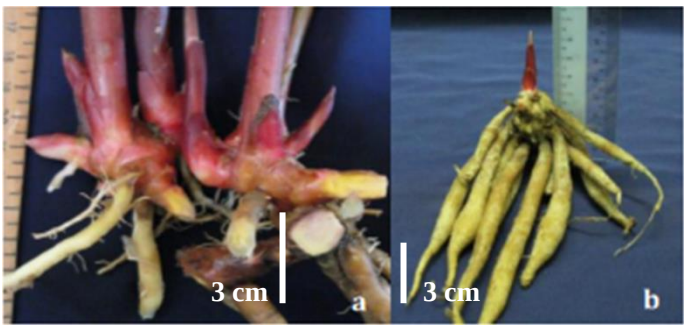

Figure 2.2: *B. rotunda*. **A:** rhizome part. **B:** tuber sprout from the rhizome part (Yusuf, 2011c).

2.2.3 Medicinal properties of *B. rotunda*

Various medicinal properties have been reported in *B. rotunda* (Table 2.1). Its ethnomedicinal usage has drawn the attention of scientists to further investigate its medicinal properties. In recent years, several compounds have been successfully identified from the rhizomes of *B. rotunda*, including boesenbergin A, cardamonin, pinostrobin, pinocembrin, pinostrobin chalcone, panduratin A, rubranine, alpinetin, sakuranetin, uvangoletin and 4-hydroxypanduratin A (Ching *et al*., 2007; Tan *et al.*, 2012a; 2012b). These compounds have been reported to exhibit antioxidant, antiparasitic, antigardial, antiulcer, antibacterial, antimicrobial, antifungal, antiviral, anti-inflammatory, antitumour/anticancer, antileukemia, antimutagenic and anti-tuberculosis activities (Tan *et al.*, 2012a; 2012b). In nature, these compounds are present in low quantity and require manipulation of complex metabolic pathway to enhance their production. Thus, a tissue culture system is essential to establish the cells that are amenable for metabolite engineering in order to exploit important metabolites for industrial purposes.

Table 2.1: Medicinal properties identified from the rhizomes of *Boesenbergia rotunda*.

Medicinal Properties	References
Oral diseases, colic and gastrointestinal disorder, diuretic, dysentery, inflammation and aphrodisiac	Saralamp *et al*. (1996) Chomchalow *et al*. (2006) Ching *et al*. (2007) Sudwan *et al*. (2007)
Antioxidant activity and neuroprotective effects	Shindo *et al*. (2006)
Anti-inflammatory activity	Tuchinda *et al*. (2002) Boonjaraspinyo *et al*. (2010) Isa *et al*. (2012)
Anti-mutagenic	Trakoontivakorn *et al*. (2001)
Anti-cancer activity	Kirana *et al*. (2007) Ling *et al*. (2010) Isa *et al*. (2012)
Anti-dermatophytic activity	Bhamarapravati *et al*. (2006)
Antibacterial activity	Voravuthikunchai *et al*. (2005) Bhamarapravati *et al*. (2006)
Chemopreventive and anti-*Helicobacter pylori* activities	Bhamarapravati *et al*. (2003)
Anti-dengue 2 virus NS3 protease	Tan *et al*. (2006) Frimayanti, (2011/2012)
Anti-feeding activity against larvae of *Spodoptera littoralis*	Stevenson *et al*. (2007)
Inhibitory effect on tumor necrosis factor -(TNF-)-induced cytotoxicity in L929 cells	Morikawa *et al*. (2008)
Antiviral effects	Sun *et al*. (2002)
Anti-ulcer activity	Tan *et al*. (2006) Abdullah *et al*. (2009) Abdelwahab *et al*. (2011)
Anti-HIV protease	Tuchinda *et al*. (2002) Tewtrakul *et al*. (2003a; 2003b)
Protective against induced cell injury	Sohn *et al*. (2005)
Fertility improvement	Yotarlai *et al*. (2011)

2.3 Suspension culture

2.3.1 Introduction

Suspension culture can be obtained from callus tissue by introducing into a liquid culture medium placed on a gyrator where the cells uniformly dispersed to form homogenous cells (Mustafa *et al*., 2011). The newly formed cells propagate in liquid culture medium and form cluster and clump together. The suspension cultures are sieved regularly to maintain only fine cells in cultures. In theory, the totipotent cells are able to regenerate into plant and synthesise natural compounds (Mustafa *et al*., 2011). A good suspension culture produces a high portion of single cells and little aggregation of clump cells. Friable and white callus (large and translucent) is an ideal source to produce fine cells in suspension culture compared to compact callus. This is because friable callus are amenable to cells separation.

Cells produced from suspension culture are grown more rapidly and showed higher cell division rate compared to callus cultured on solid medium. Besides propagating plantlets rapidly, suspension cultures also provide lower production cost (Aitken-Christie, 1991). Suspension culture is free of external constraints and chemicals associated with growth centre where cells are able to divide in all directions with ease and randomness of cell division. These provide an advantage when many cell generations are required or for more uniform treatments on cells (Philips *et al.*, 1995).

The suspension culture medium normally consists of 2 types of growth regulators, auxins and cytokinins. An optimal combination of both growth regulators varied depending on the plant genotype. High ratio of auxin to cytokinin usually induces higher cell division. Suspension cultures can be used for studies in plants physiology, biochemistry and molecular biology. It provides single embryogenic cells and somatic embryos suitable for gene transfer and transformation (Iantcheva *et al.*, 2006).

2.3.2 Growth cycle of suspension culture

Suspension cells grow slowly during the initial growth period also known as lag phase where aggregate cells dispersed into culture medium readily initiate cell division unlike single cells. Biomass increased as the cell continuously divides and enlarge during subsequent incubation, which is known as the exponential phase. This condition outlast until the growth stops due to either exhaustion of nutrients supply or over accumulation of metabolite toxics in the culture medium, which is known as stationary phase. Cell aggregations and its maximum cell density are achieved during this phase (Mustafa *et al*., 2011).

In order to maintain active cell division in the suspension cultures, sub-culture process is necessary where a small portion of the cells from the stationary phase is transferred to a new culture medium (Mustafa *et al*., 2011). The cells in suspension culture are either homogenous (genetically identical) or heterogenous (genetically vary). The heterogenous group of cells can be avoided by continuously sub-culturing into fresh medium during early stationary phase.

2.3.3 Advantages and applications of suspension cultures

Plant suspension cultures offer several advantages over *in vitro* whole plant cultures. Suspension culture is defined as rapid proliferation of cells in liquid medium and to avoid repeated generation of plants from seeds by periodic subculture. It has been broadly applied to generate plant biomass with low cost and less space (Castellar *et al*., 2011; Yusuf *et al*., 2011a; 2011b). Suspension cultures also provide a stable platform to introduce transgene into crops due to presence of homogenous cell production in comparison to whole plant cultures resulting in consistent product output and stable transgene lines (Shih and Doran, 2008; Boivin *et al*., 2010; Xu *et al*., 2011). Besides, it can be used to study physiology, biochemistry and molecular biology changes in plants for a short period (Shih and Doran, 2008). Production of secondary metabolites using cell cultures has been reported in many plant species (Mustafa *et al*., 2011; Valluri, 2009; Cai *et al*., 2011).

In recent years, plant suspension cultures have been used as a biofactory to produce pharmaceutical compounds, such as taxol, glucocerebrosidase and antibody against Hepatitis B, at low cost and safe level (Lau and Sun, 2008; Basaran and Cerezo, 2008; Xu *et al*., 2011; Huang and McDonald, 2012). Furthermore, this technology can be easily scaled-up to produce more cells or plantlets using bioreactor.

2.4 Protoplast

2.4.1 Introduction

Protoplast is a complete single cell without cell wall, bounded only by plasmalemma. Physiologically, protoplast is not simply a ‘cell without cell wall’ as during the cell wall removal process it also affects the cell metabolism and cell ultrastructure such as microfilament, microtubule and actin filament. Without cell wall, permeability of the cell membrane is compromised and caused some solutes leakage from the protoplast. The isolated protoplasts, irrespective of the environment, start to initiate the new cell wall synthesis within few hours to produce single-walled cell.

Protoplasts isolation from leaves always includes the removal of the lower epidermis before enzyme incubation to allow permeability to the cell. Protoplasts from calluses and suspension cultures were frequently isolated during the log phase of the growth cycle (Jude and Fred, 2011). This is because the secondary products such as lignin is formed in cell wall as the cultures mature, subsequently render the cell wall degradation by enzymes. With suitable enzyme cocktail and osmoticum level, most plant tissues and organs can produce protoplasts.

Protoplasts are isolated either through mechanical or enzymatic technique. Mechanical isolation technique was not popular due to extremely low yield of isolated protoplast but using enzyme method produced contrary result (Cocking, 1960). With the success of protoplast isolation technique, recovery and regeneration ability of isolated protoplast also play an important role in propagation and genetic transformation.

Nagata and Takebe first succeeded in demonstrating the protoplast regeneration ability in Tobacco mesophyll cell (Nagata and Takebe, 1971). Since then, many reports on novel protoplast-to-plant systems for genetic manipulation were published (Guo *et al*., 2007; Wang *et al*., 2008; Hassanein *et al.*, 2009; Kothari *et al.*, 2010, Sun *et al*., 2011).

Gene transfer technology is commonly used for crop improvement, production for novel proteins and compounds. Many of these transgenic plants already been commercialised. Due to resistance in public acceptance toward recombinant DNA technologies, interests on protoplast technology such as somatic hybridisation, cybridisation, and protoclonal variation studies may revive.

2.4.2 Protoplast isolation methods

Enzymatic isolation technique produces high protoplast yield and less damaging to target cells (Davey *et al.*, 2000; 2003). This technique could either be carried out in one-step or two-step procedures. In one-step isolation, a mixture of enzymes (e.g. cellulase and macerozyme) was used on the target plant tissue. The optimal composition of enzymes mixtures (Power and Chapman, 1985) and isolation protocol varied for different plants. In two-step isolation method, protoplasts were isolated stepwise using single enzyme type. Initially, individual cells were separated by degrading the middle lamellas using maceratic enzymes (macerozyme and macerase), and subsequently the protoplasts were released by degradation of the cell wall using cellulases (cellulase Onozuka R-10, cellulysin). Two-step isolation method involved shorter enzyme treatment period compared to one-step isolation. Enzymatic isolation technique isolated only parenchymal cells with unlignified cell walls. This is because lignified cell walls prevented the action of enzymes on targeted cells.

Protoplast could be isolated from a wide range of species. However, only viable ones are potentially totipotent. Theoretically, each protoplast is able to recover to form new cell wall and mitotically divide to form daughter cells under suitable chemical and physical stimuli. It also can regenerate to produce fertile plants using tissue culture technique. To date, protocols for protoplast-to-plant systems are available for several plant species (Zhou *et al.*, 2008).

2.4.3 Factors influencing the protoplast isolation

Numerous factors have been reported to influence the protoplast isolation, including the source of tissues (e.g. leaves and cell suspension), composition of cell walls and enzymes used, enzyme incubation period, pH of the enzyme solution, speed of agitation and osmotic pressure (Sinha *et al*., 2003; Davey *et al.*, 2005; Zhou *et al.*, 2008; Kativat *et al*., 2012; Silva Jr., 2012).

Protoplasts can be isolated from different tissues and organs (Zhai *et al.*, 2009), such as leaves, shoot apices, roots, embryos, pollen grains, calli and suspension cells. The yield and viability vary according to the genotype and explants used (Silva Jr., 2012). The physiological conditions, plant age, environmental and seasonal conditions of target plants can also influence the success of protoplast isolation (Davey *et al*., 2005; Pongchawee *et al*., 2006; Raikar *et al*., 2008). Thus, *in vitro* plants grown under controlled conditions are commonly used (Bhojwani and Razdan, 1983; Lord and Gunawardena, 2010).

Physical conditions, such as temperature, incubation period and ratio of enzyme cocktail to target plant tissue can influence the yield and viability of isolated protoplasts. Incubation time plays crucial role in protoplast isolation and it is highly dependant on plant species. Inappropriate incubation time can result in incomplete digestion of cell wall and over-digestion of protoplast. The enzyme incubation time varies from short- (2 – 6 hours) to long-term period (16 – 24 hours) in either light or dark conditions.

Besides concentrations and pH of enzyme cocktail, purification of isolated protoplasts from cell wall residuals, sub-protoplasts (damaged protoplasts) and enzyme cocktail is important for subsequent protoplast culture process. This can be done by repeating floatation purification (filtration, centrifugation and washing) (Landgren, 1978; Jude and Fred, 2011). Agitation during enzyme incubation aids in increasing the protoplast yield (Dědičová, 1995; Silva Jr., 2012).

Besides, osmotic pressure of the solution for isolation and culture media are very important to avoid the protoplasts from bursting. Osmotic conditions also indirectly influence the yield and viability of isolated protoplasts as well as subsequent protoplasts culture process (Silva Jr., 2012). The osmotic pressure of enzyme cocktail, washing solution and culture medium is adjusted through incorporation of mannitol, sorbitol, glucose and sucrose. Stability of protoplasts is better in slightly hypotonic conditions compared to isotonic conditions.

Plating density (number of protoplasts per mL) can influence the division of protoplasts and microcalli formation. Ideally, density between 10^4 - 10^6 (Davey *et al*., 2005) protoplasts per mL is the optimal plating density in many plants. High and low plating density may inhibit cell division and colony formation (Davey *et al*., 2005).

2.4.4 Protoplasts culture

During protoplast culture, regeneration of cell wall is crucial prior to cell division. The ability to regenerate cell wall is dependent on the use of suitable culture media which requires osmotic protectant before new primary walls are regenerated to counteract with turgor pressure caused by the cytoplasm (Yang *et al*., 2008). Culture medium, light intensity and temperature play an important role in the success of protoplasts culture (Dědičová, 1995).

Early stages of cell wall synthesis start with extensive plasmalemma folding and accumulation of pectin-like substances in vesicles in the peripheral layer of cytoplasm. This process does not require any new RNA or protein synthesis as the residual protein and endogenous hormone are sufficient to initiate cell wall formation. The first formed envelope is structurally amorphous and has pectins deposit on it. A single layer of cellulose fibrils will subsequently be laid on the protoplast surface after a few days, followed by a formation of normal cellulose matrix (Burgess and Fleming, 1974).

Protoplast, like cell suspension, has an optimum plating density to undergo division. The common plating density used is 10^4 - 10^5 protoplasts per mL in many plants. The ability of plated protoplasts to form cell colonies or plating efficiency is scored after a certain period. The osmoticum level in culture medium has to be reduced gradually as the division proceed.

There are different types of protoplast culture medium such as liquid, semi-solid and solid medium. In addition, liquid and solid medium can be used together where protoplasts are embedded inside solid medium and cultivated in liquid medium (Erikson, 1986). Liquid medium is more preferred compared to solid medium as the osmotic pressure in culture medium can be easily regulated. During the protoplasts culture, the osmotic pressure of culture medium is lowered following the first cell division after cell wall formation to enable continuous cell division (Kao and Michayluk, 1980).

Many types of basal media, such as Murashige and Skoog (MS) (1962) and B5 (Gamborg *et al*., 1968) formulations, with additional of osmotic protectant such as mannitol (non-metabolisable sugar alcohol) and plant growth regulators were used for sustained protoplast growth.

2.4.5 Protoplast regeneration

The regeneration process from protoplasts to plants can be divided into 3 stages (Nagata and Takebe, 1971). During initial stage, protoplasts in suitable medium can form new cell wall and initiate first cell division until formation of microcalli. During differentiation stage, with suitable medium (high cytokinins and low auxins) shoots develop from macrocalli. During rooting stage, usually medium without growth regulators promote roots formation from regenerated plant.

Protoplasts not only can reform its cell wall and undergo division to form macrocalli, but also has the ability to regenerate into whole plant. Whole plant regeneration is not restricted to either monocot or dicot, haploid or diploid, or source of protoplasts isolated. Plants regenerated from protoplasts exhibit normal plants traits with high degree of fertility. However, a small percentage may show morphological abnormalities (aneuploidy and polyploidy).

Formation of new cell wall varies from a few hours to days of protoplast culture, where protoplasts start to lose their spherical shape, followed by division to form cell colonies after a few weeks and eventually macrocalli formed. Most protoplasts have the ability to undergo division, while some were not able to do so (Bhojwani and Razdan 1983). Successful protoplasts regeneration may be determined by genotype, culture media, conditions and methods (Roest and Gillisen, 1989).

3.0 Materials and methods

3.1 Plant materials and maintenance of cultures

B. rotunda suspension culture established after 6 months was obtained from the Plant Biotechnology Research Laboratory, University of Malaya, Malaysia. The callus cultures were induced from rhizome buds according to Tan *et al.* (2005). 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 (IAA), 2 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), 1 mg/L 1-naphthylacetic acid (NAA), 30 g/L sucrose and 2 mg/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. The cultures were incubated at 25 ± 2 ℃ under continuous shaking condition of 80 rpm with a 16-h light and 8-h dark photoperiod. The cells were subcultured every 14 days by transferring 10 mL of 10 % (v/v) settled cells into a 250 mL conical flask and made up to a final volume of 50 mL with fresh liquid MS medium (Appendix A; Table 2). The medium was adjusted to pH 5.8 ± 0.2 and autoclaved at 121 ℃ for 20 min.

3.2 Optimisation of factors affecting cell suspension cultures growth

To optimise the conditions of cell growth, cell suspensions were inoculated in liquid MS medium supplemented with different concentrations of 2,4-D (Sigma, USA) (0, 2, 4, 8 and 16 mg/L) and sucrose (Systerm, Malaysia) (0, 1.5, 3, 4.5 and 6 % w/v). To determine the effect of sonication on cell growth, cell suspensions were sonicated at different times (0, 30, 120, 300 and 600 s) in a water bath sonicator (Elmasonic P 30 H; Elma, USA). Settled cell volume (SCV) was measured in 3-day intervals until 27 days and the specific growth rate (μ) of each treatment was calculated using formula: $\mu = [\ln (\text{Final} / \text{Initial})] / \text{Time}$. All cultures were incubated at 25 ± 2 ℃ under a 16-h light and 8-h dark photoperiod with a light intensity of 1725 lux provided by cool white fluorescent light.

3.3 Isolation of protoplast

The protoplast was isolated according to the protocol of Geetha *et al*. (2000) with modifications. Ten mL of suspension culture containing 20 % (v/v) settled cells were incubated with an equal volume of filter sterilised enzymes (cellulase and macerozyme) in different concentrations and combinations (Appendix A; Table 3). Enzymes were filter sterilised using 0.2 µm milipore filter (Sartorius Stedim Biotech, Germany). The mixture was then incubated at 25 ± 2 ℃ for 5, 24 or 48 h under continuous shaking condition of 50 rpm on a rotary shaker (Hotech Shaker Model 723, Taiwan). 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* (Minor Centrifuger, USA). The sediment was washed with protoplast washing medium (CPW13M) (Appendix A; Table 4) 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. The mixture was floated on 8 mL protoplast floatation medium (CPW21S) (Appendix A; Table 4) by gently pipetting the mixture on CPW21S without mixing. 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. 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 maintenance of protoplasts integrity and subsequent protoplast counting.

The number of protoplasts isolated was counted using a Fuchs-Rosenthal haemocytometer counting chamber (Figure 3.1). It consists of 16 big squares with one mm^2 areas each and orientated by triple lines with a volume of 0.2 mm^3 (2×10^{-4} mL). Each big square is sub-divided into 16 small squares with a depth of 0.2 mm and an area of 6.25×10^{-2} mm^2, (volume for one small square is 1.25×10^{-2} mm^3). The number of protoplast per mL was calculated using the following formula:

$$\frac{\text{Average protoplast number in one big square}}{2 \times 10^{-4}\ \text{mL}}$$

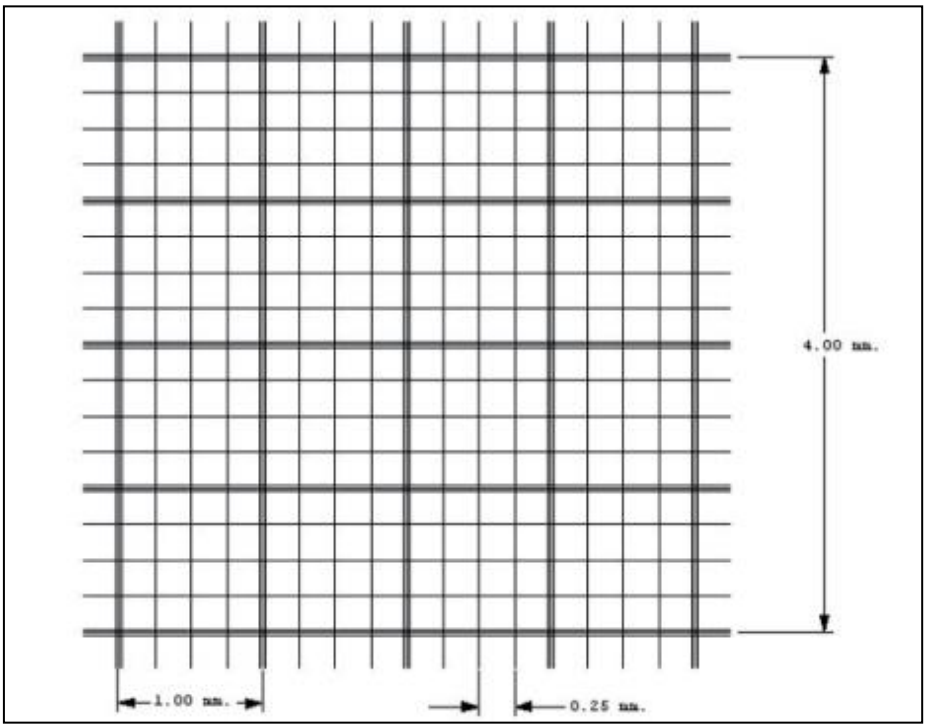

Figure 3.1: Fuchs Rosenthal Counting Chamber (Science service, 2013, July 9).

3.4 Calcofluor white M2R and fluorescein diacetate (FDA) staining

Calcofluor white M2R (Sigma, USA) powder was dissolved in distilled water and the solution was adjusted to pH 10-11 with 1 N sodium hydroxide (NaOH) to a final concentration of 3.5 mg/mL, whereas Fluorescein Diacetate (FDA) powder was dissolved in acetone with a final concentration of 5 mg/mL. The formation of cell wall was determined using calcofluor White M2R (Fluorescent Brightener 28, Sigma, USA) by adding 20 µL calcofluor white M2R into 0.5 mL CPW13M containing protoplasts. The mixture was incubated for 10 min and examined under UV florescence microscope (Axiovert 10, Zeiss, Germany). The viability of isolated protoplasts was determined using FDA stain (Sigma, USA) by adding 20 µL FDA into 0.5 mL CPW13M containing protoplasts. The mixture was incubated for 15 min and examined under UV florescence microscope.

3.5 Recovery of protoplasts

Protoplast density was adjusted to 1-5 $\times$ 10^5 protoplast per mL using CPW13M and cultured in 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; Appendix A; Table 5) 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; Appendix A; Table 5) 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.

3.6 Statistical analysis

The data collected were analysed statistically by one-way analysis of variance (ANOVA) followed by Duncan's multiple-range test at a significance level of $p < 0.05$ using Statistical Package for the Social Science (SPSS) version 16.0.

4.0 Results and discussion

4.1 Suspension culture

It is crucial to optimise the growth of suspension cultures in order to obtain high biomass of cells that can be subsequently used for protoplast isolation. Therefore, in this study, the effects of sonication and supplementation of different concentrations of 2,4-D and sucrose on the growth of cell suspension cultures were investigated.

4.1.1 Effect of 2,4-D treatment on cell growth

The growth of *B. rotunda* cell suspension cultures under the influence of plant growth regulator was investigated (Figure 4.1). Supplementation of 2,4-D in the MS medium did not accelerate cell growth, whereas 2,4-D-free MS medium (days 6 to 18) produced the highest growth rate ($\mu = 0.0688$) compared to other treatments. The specific growth rate of cultures inoculated in MS medium containing 2,4-D at 4 mg/L and 8 mg/L were not significantly ($p < 0.05$) different compared to the control (Table 4.1), whereas 2,4-D at 2 mg/L and 16 mg/L were significantly ($p < 0.05$) lower than the control .

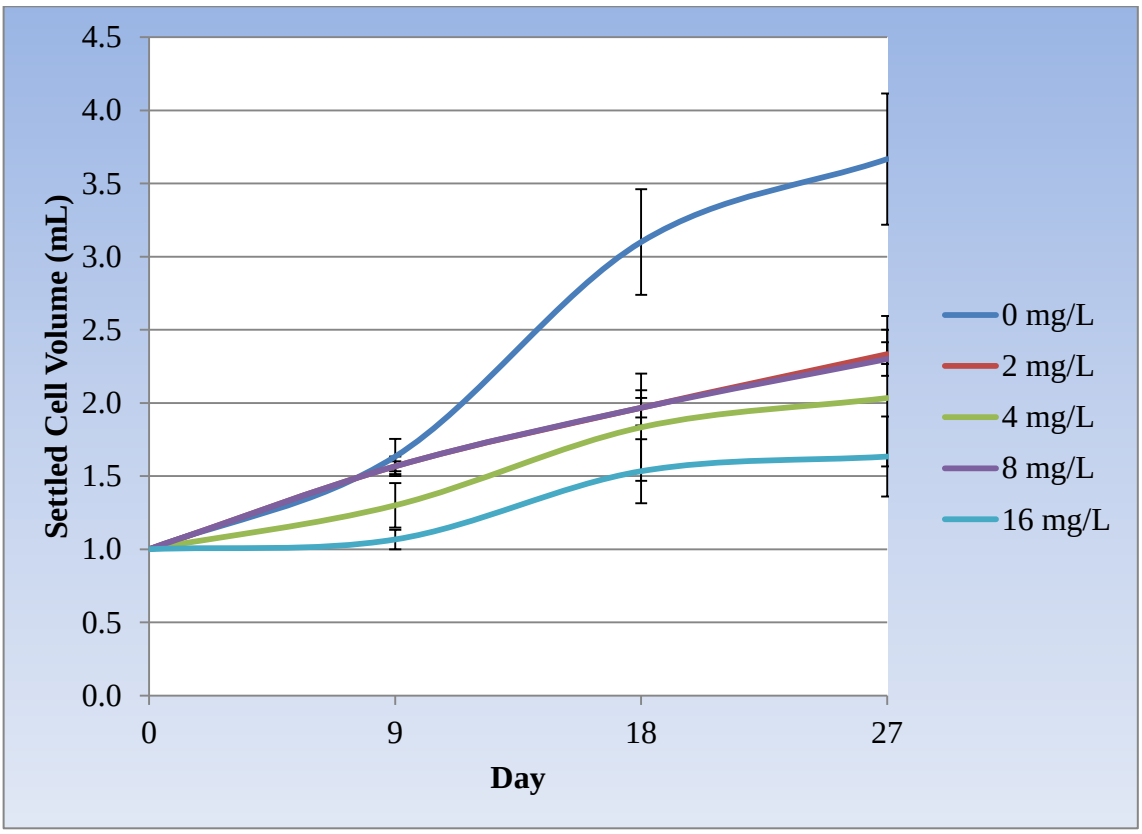

Figure 4.1: Effect of different concentrations of 2,4-D on cell density.

Table 4.1: Effect of different concentrations of 2,4-D on cell suspension growth rate from day 6 till day 18.

2,4-D (mg/L)	Specific growth rate (µ/d)
0	0.0688 ± 0.0038 [a]
2	0.0269 ± 0.0100 [b]
4	0.0352 ± 0.0290 [ab]
8	0.0435 ± 0.0133 [ab]
16	0.0311 ± 0.0270 [b]

2,4-D has been considered as a specific limiting factor in plant growth. Their presence within or outside the cells in a certain amount might cease the cell division (Leguay and 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). This might be due to the phytotoxicity effect of 2,4-D in the suspension culture and thus, render the cell growth (Tewes *et al*., 1984). Although 2,4-D is widely used for callus induction, however, it exhibits greater inhibitory effect to long-term suspension cultures 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 higher frequency of browning was recorded. Since the plant cells also contain endogenous growth regulators, therefore continuous growth of suspension culture without 2,4-D was possible (Jimenez *et al*., 2005).

4.1.2 Effect of sonication on cell growth

Sonication is a physical stimulus that may be used to stimulate biological activities (Schläfer *et al*., 2000), including shoot regeneration, seeds germination and plant growth from recalcitrant tissues (Godo *et al*., 2010; Shin *et al*., 2011). In this study, cell suspensions were sonicated at different times (0, 30, 120, 300 and 600 s) in a water bath sonicator. All sonicated suspension cultures exhibited negative growth rate, whereas the suspension cultures without sonication recorded positive growth at 0.0264 SCV/day (Table 4.2; Figure 4.2).

Table 4.2: Effect of various sonication times on cell suspension growth rate from day 6 till day 18.

Sonication (s)	Specific growth rate (μ/d)
0	0.0269 ± 0.0100^{a}
30	-0.0080 ± 0.0139^{bc}
120	-0.0279 ± 0.0060^{b}
300	-0.0026 ± 0.0046^{c}
600	-0.0225 ± 0.0195^{bc}

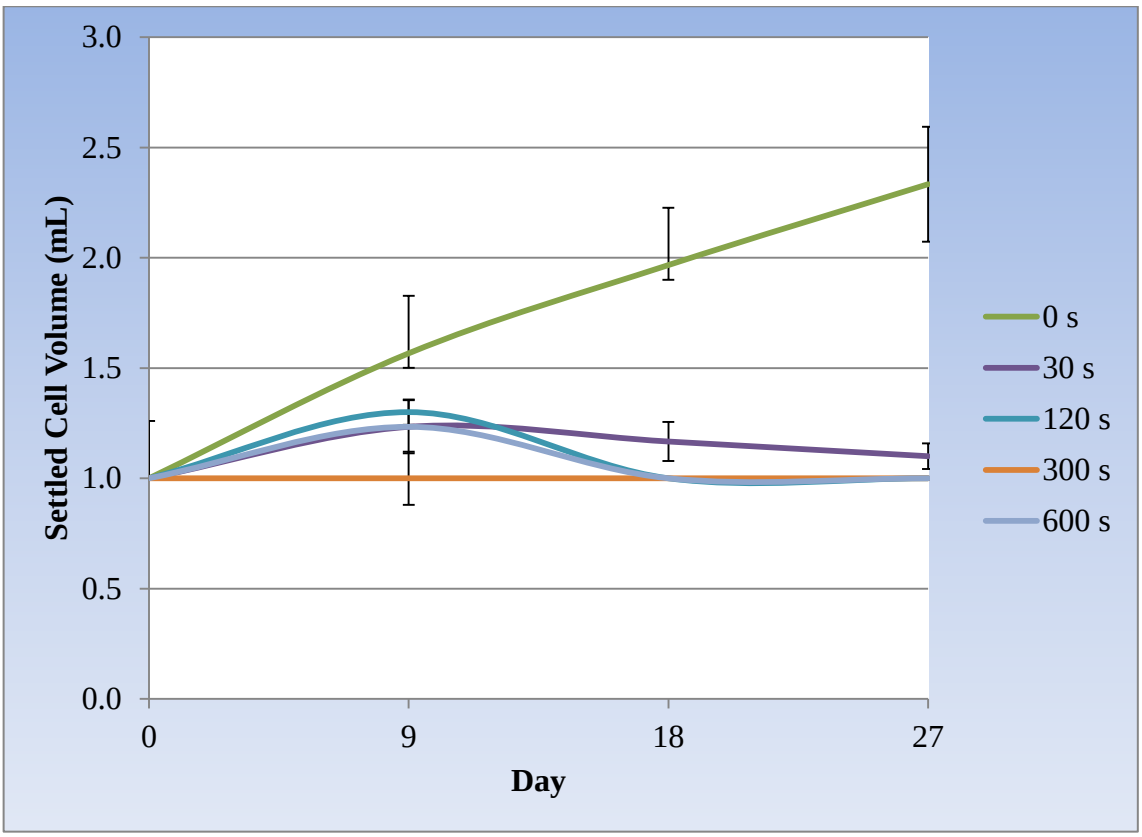

Figure 4.2: Effect of different sonication times on cell density.

Cells remained viable in non-sonicated treatment until 27 days of culture. The sonicated cultures were viable on the first day of treatment and appeared cloudy (Figure 4.3). Occurrence of non-viable cells might be due to toxicity and insufficient nutrients supply. The media of the sonicated suspension cultures appeared green fluorescein under blue light probably due to the released cell components, such as protein content and intracellular matrix, from damaged sonicated-cells (Figure 4.3) (Koch *et al.*, 2007). All sonication treated cells were not viable after 27 days, except for those exposed to 30 s sonication as indicated by FDA staining (Figure 4.4B).

The finding obtained from this study was in agreement with the study carried out by Bohm *et al*. (2000), where the viability of *Petunia* hybrid suspension culture was decreased to 35 % under standing-wave condition. In contrast, Wu and Lin (2002) reported a significant drop in the viability of the *Panax ginseng* suspension culture after exposure to ultrasound, however, it gradually recovered after 2-3 days with higher ultrasound power and longer exposure period. The bioeffects of ultrasound on suspension cells are mainly due to mechanical stress introduced by ultrasound-induced fluid motion as well as the hydrodynamic events (Miller *et al*., 1996). According to Bohm *et al*. (2000), cellular viability under sonication treatment depended on several aspects, including acoustic energy density, exposure time, and mechanical properties of the cells determined by the cell age.

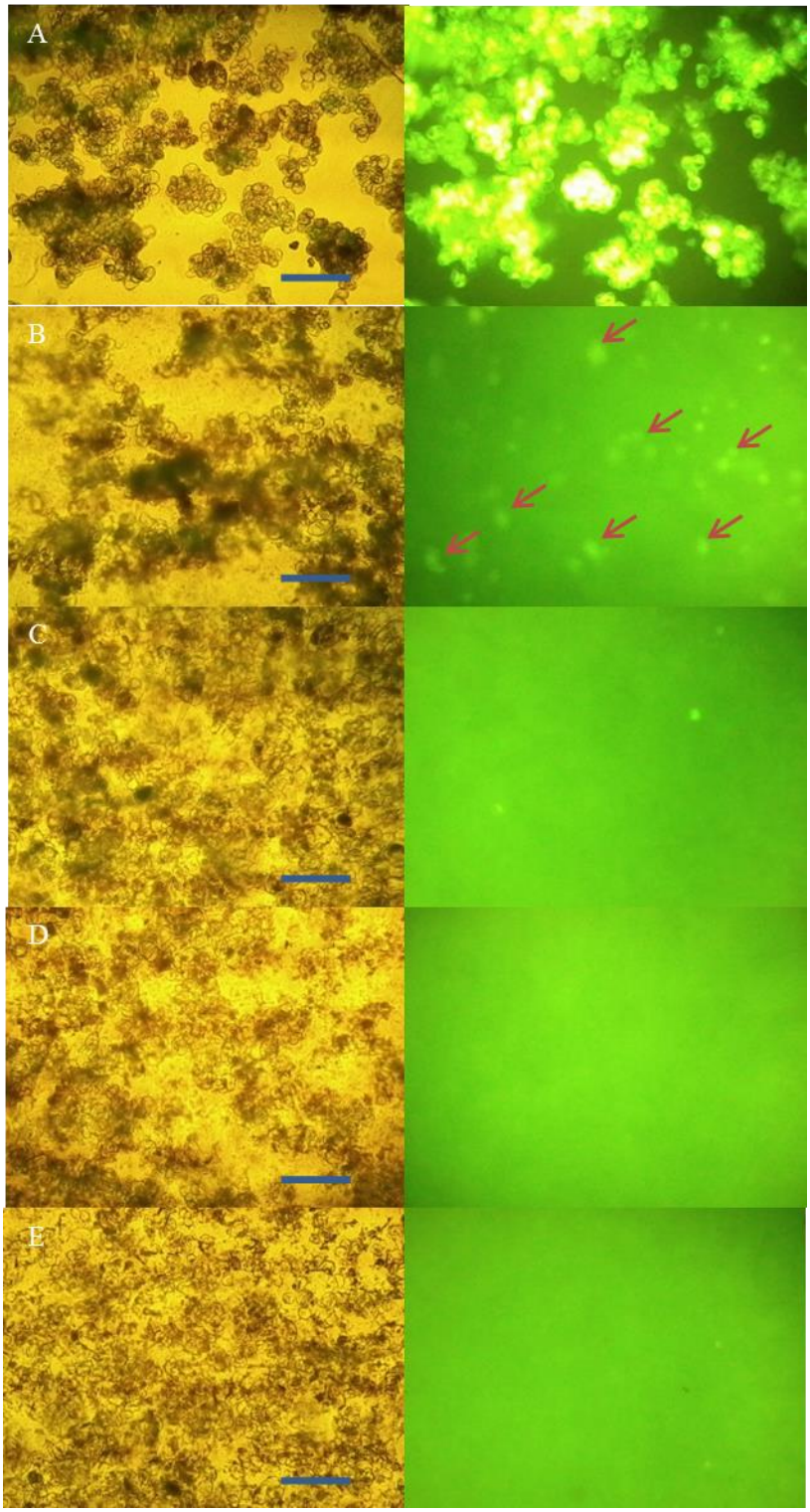

Figure 4.3: *B. rotunda* suspension cells with sonication and FDA test (green) at first day. **A:** 0 s sonication treatment, **B:** 30 s sonication treatment, **C:** 120 s sonication treatment, **D:** 300 s sonication treatment and **E:** 600 s sonication treatment. Red arrows indicate viable cells after 30 s sonication treatment. Bar indicates 0.25 mm.

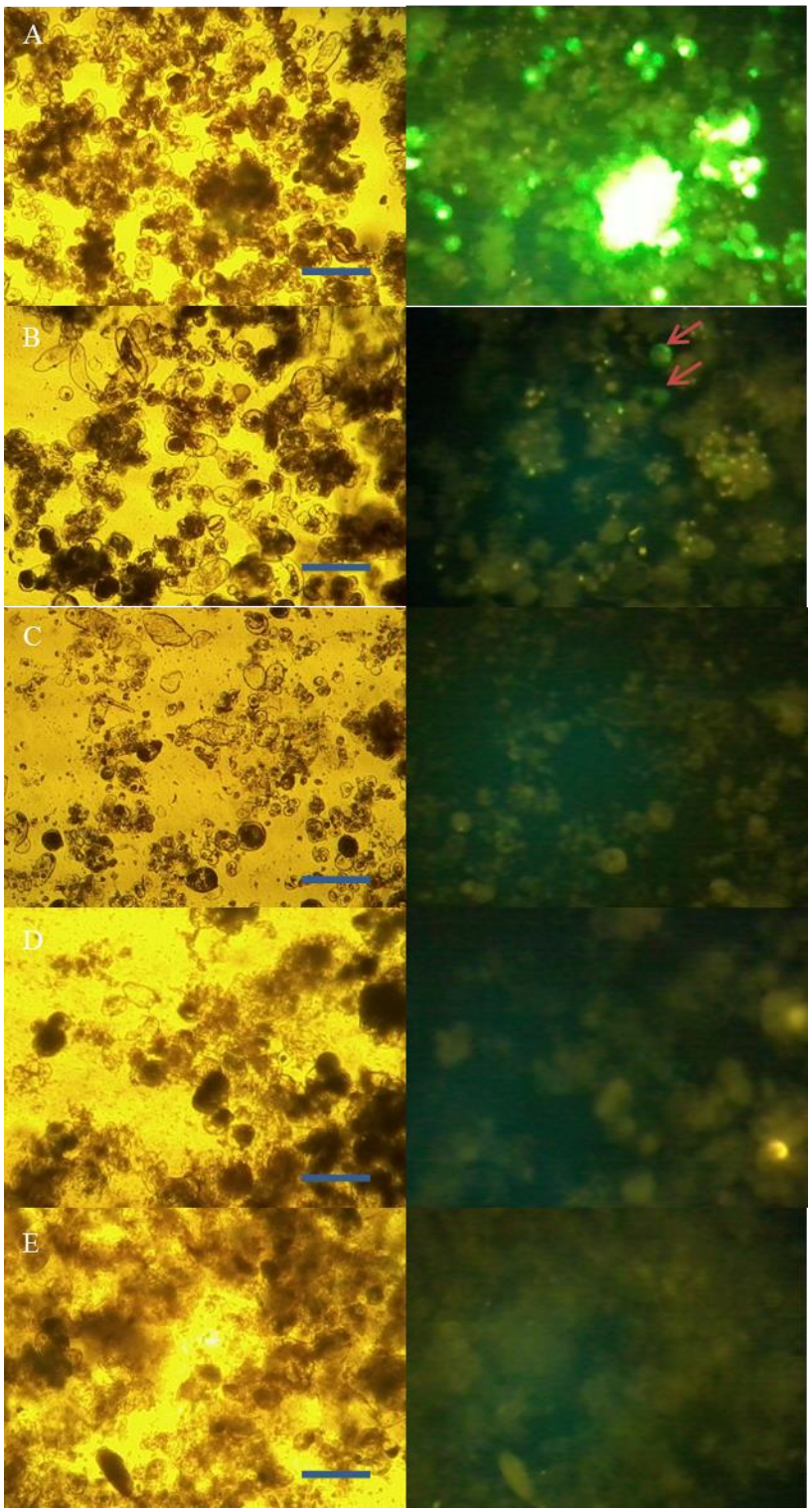

Figure 4.4: *B. rotunda* suspension cells with sonication and FDA test (green) after 27 days (last day). **A:** 0 s sonication treatment, **B:** 30 s sonication treatment, **C:** 120 s sonication treatment, **D:** 300 s sonication treatment and **E:** 600 s sonication treatment. Red arrows indicate viable cells. Bar indicates 0.25 mm.

4.1.3 Effect of sucrose on cell growth

The effects of different concentrations of sucrose, a carbon source for maintenance of suspension cultures, were investigated. The results indicated that the growth of suspension cultures was influenced by sucrose. In general, low cell growth rate was recorded in MS medium without sucrose compared to the medium containing sucrose (Figure 4.5).

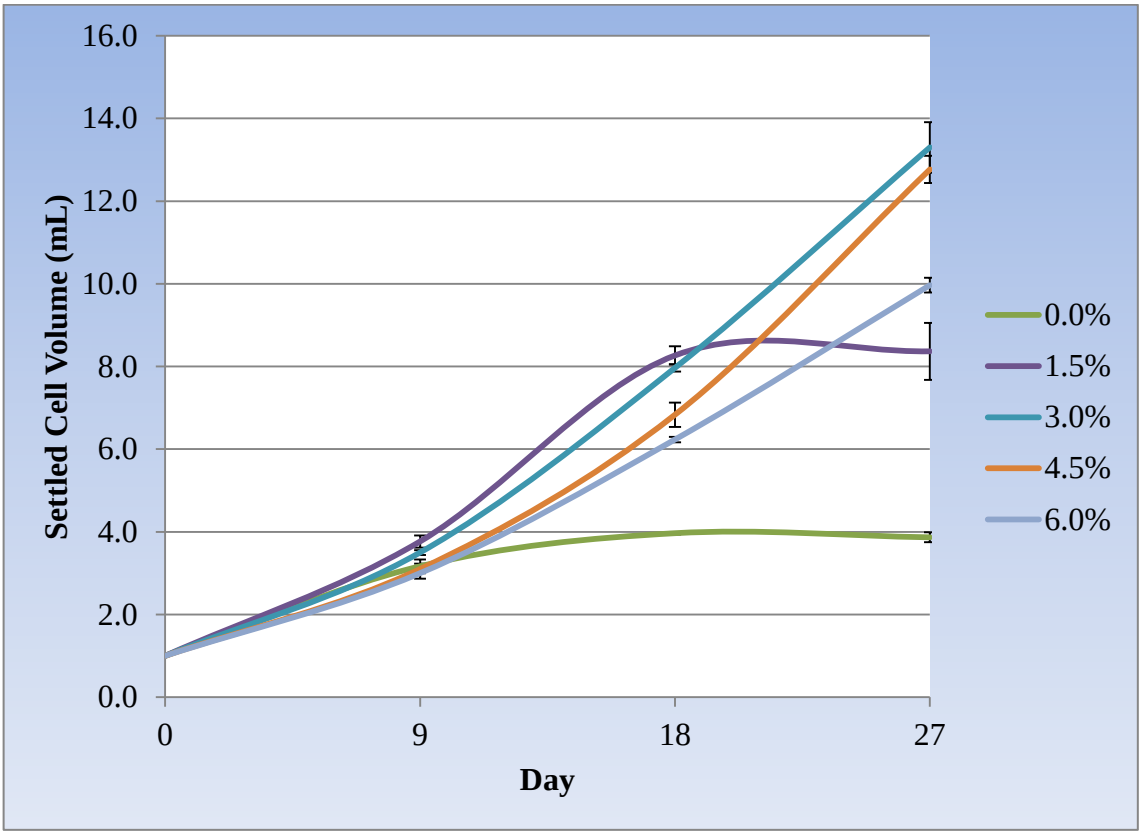

Figure 4.5: Effect of different sucrose concentrations on cell density.

The highest specific growth rate (μ) of cells was observed in the media containing 1.5 % and 3 % sucrose with 0.1155 ± 0.0061/day and 0.1125 ± 0.0037/day respectively (Table 4.3). However, medium supplemented with 3 % sucrose showed the highest final SCV at day 27 compared to other concentrations tested (Figure 4.5). Similar result was observed by Abdullah *et al*. (1998), who reported that culture medium containing 3 % sucrose successfully improved the cell growth of *Morinda elliptica* suspension cultures.

Table 4.3: Effects of different concentrations of sucrose on cell suspension growth rate from day 6 till day 18.

Sucrose (%)	Specific growth rate (μ/d)
0.0	0.0557 ± 0.0021 [a]
1.5	0.1155 ± 0.0061 [b]
3.0	0.1125 ± 0.0037 [b]
4.5	0.1010 ± 0.0003 [c]
6.0	0.0922 ± 0.0075 [d]

The growth rate of cell suspension culture was significantly decreased to 0.1010 ± 0.0003/day and 0.0922 ± 0.0075/day when cultured in MS medium containing 4.5 % and 6 % sucrose, respectively. High concentration of sucrose might affect the water content in the suspension cells due to osmotic pressure (Ho *et al*., 2010) and thus, affect the cell growth. This high osmotic pressure has been reported to inhibit nutrients uptake (Lee *et al*., 2006) and halt 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). Cell suspension cultures in MS media containing 0 % and 1.5 % sucrose did not show any continuous growth beyond 18 days of culture in contrast to 3, 4.5 and 6 % sucrose augmented media. This might be due to depletion of carbon source to support cell growth.

4.2 Isolation of protoplast

The success of protoplast isolation depends on the types and concentrations of enzymes used, incubation period and source of protoplast. Inappropriate use of enzymes and incubation time may result in either incomplete digestion of cell wall or over-digestion of protoplast. In this study, different concentrations of cellulase and macerozyme as well as their incubation times were investigated.

4.2.1 Source of protoplast

In this study, 5-day old suspension cultures in the early logarithm phase were used as a source to isolate protoplasts (Figure 4.6). Suspension cultures in this phase consist of small cells with a thin cell wall which are suitable for protoplast isolation (Mastuti *et al*., 2003; Grosser and Gmitter Jr, 2011). In this phase, suspension cultures consist of cells which are small and most probably with thin cell-walled to ease cell wall digestion. After early logarithm phase, suspension cells enlarge with large vacuole and thicker cell wall which are not suitable for high yield protoplast . Besides, isolation of protoplast from cell suspension cultures at the stationary phase remains technically challenged and may need a complex enzyme digestion as the cells start to lignify their cell wall at this stage (Schenk and Hildebrandt, 1969).

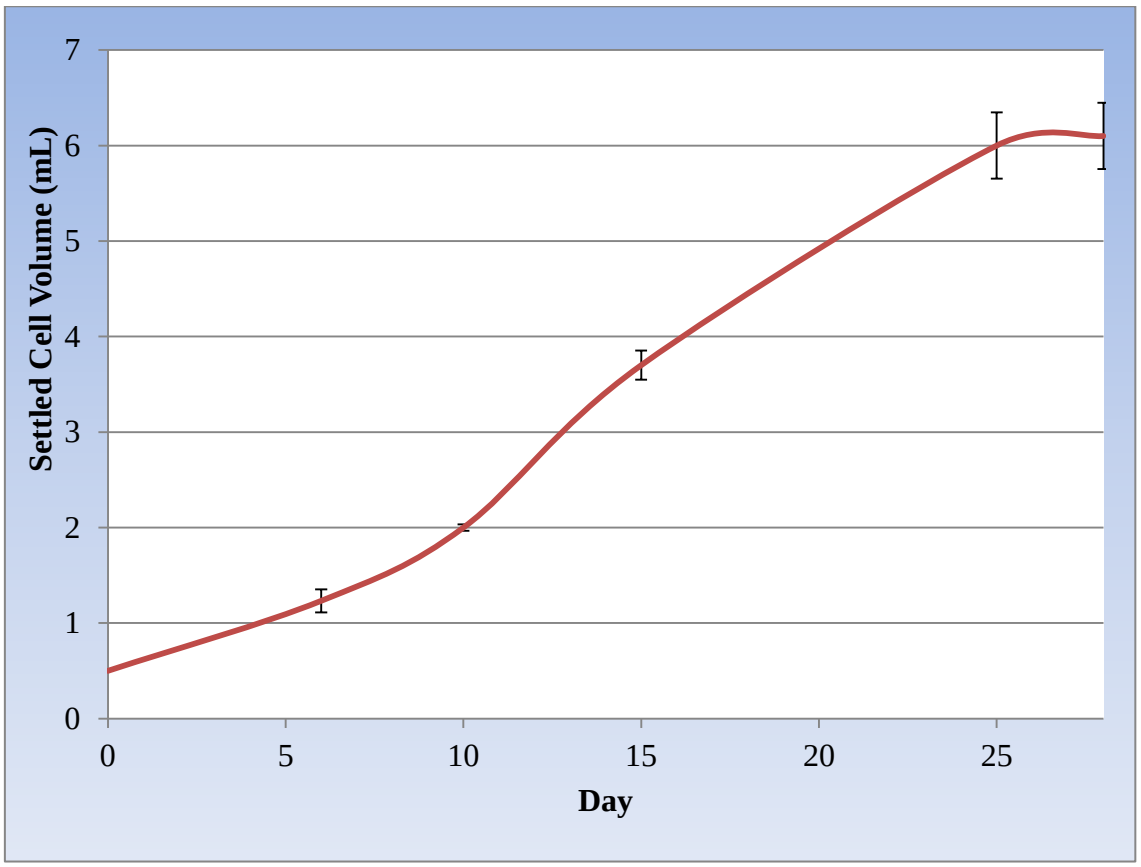

Figure 4.6: Standard growth curve for *B. rotunda* cell suspension culture.

4.2.2 Optimisation of enzyme combinations for protoplast isolation

Macerozyme and cellulase enzymes were used in different combinations and concentrations to isolate protoplasts. The highest protoplast yield ($2.20 \times 10^5 \pm 0.21$ per mL) was recorded when a combination of 2.0 % cellulase and 0.5 % macerozyme was used (Figure 4.7). Similar result was observed when the same ratio (4:1) of cellulase and macerozyme at 1 % and 0.25 % was applied, where a total of $1.96 \times 10^5 \pm 0.28$ protoplasts per mL was produced. This suggested that the ratio of cellulase and macerozyme enzymes was important to obtain a good protoplast yield. Macerozyme is commonly used in a range of 0.1 to 1 % while cellulase is between 0.5 to 5 % for isolating protoplast in many plant species (Geetha *et al.*, 2000; Mastuti *et al.*, 2003; Guo *et al.*, 2007). Successful methods using this combination have also been reported in *Zingiber officinale* Rosc. (Guo *et al.*, 2007), *Nicotiana tabacum* (Uchimiya and Murashige, 1974), *Elettaria cardamomum* Maton (Geetha *et al.*, 2000) and *Celosia cristata* L. (Mastuti *et al.*, 2003).

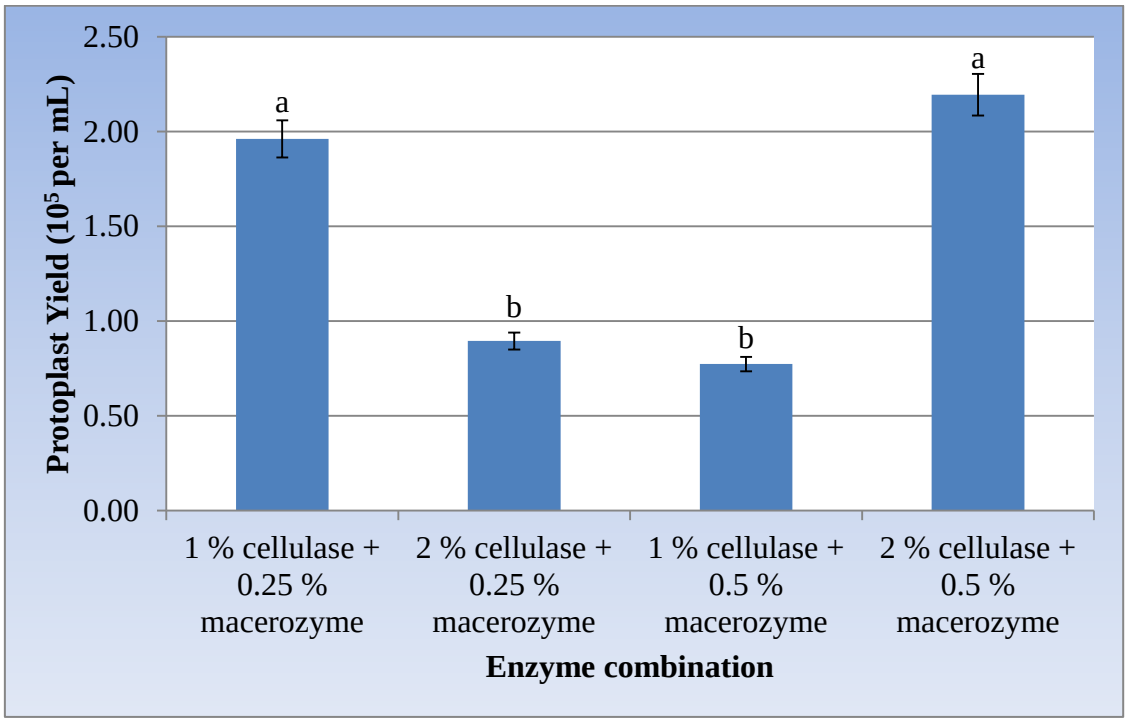

Figure 4.7: Effect of different combinations and concentrations of enzymes on protoplast yield.

Macerozyme is widely used to isolate a single cell from cell clumps or explants, whereas cellulase is used to digest the cellulose component of the cell wall from isolated single cells. The combination of different types of enzymes has been reported to be useful in isolating protoplast. This was in agreement with the study carried out by Uchimiya and Murashige (1974), where less protoplast was isolated using a single enzyme in tobacco cells due to enzyme substrate specificity (Chen *et al*., 1994).

4.2.3 Optimisation of incubation period

Optimised enzyme combination of 1 % cellulase and 0.25 % macerozyme (Section 4.2.1) was selected for subsequent experiment to determine the optimal incubation period. Three different incubation times (5, 24 and 48 h) were tested. The results revealed that cells incubated with enzymes at 24 h produced the highest protoplast yield ($1.96 \times 10^5 \pm 0.28$), whereas $0.46 \times 10^5 \pm 0.10$ and $0.35 \times 10^5 \pm 0.10$ were recorded in the cells incubated for 5 and 48 h, respectively (Figure 4.8). Differences between the incubation times 5 and 48 h were not significant ($p < 0.05$).

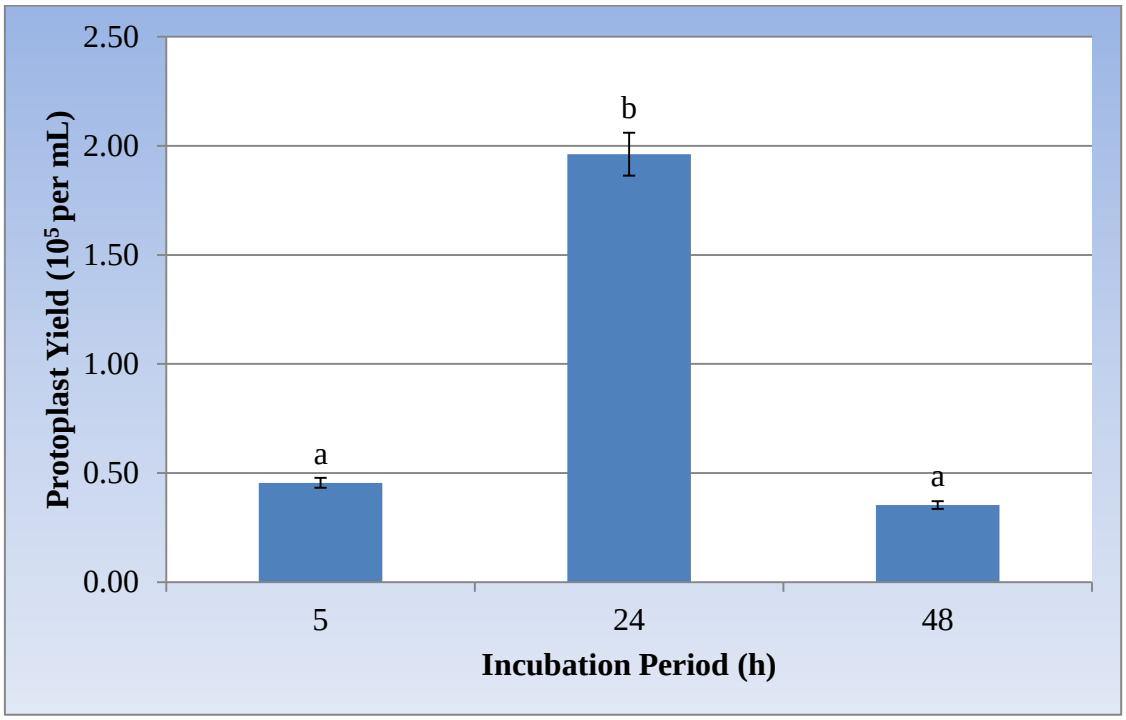

Figure 4.8: Effect of different incubation period of enzymes on protoplast yield.

Enzyme incubation period is one of the critical factors to ensure good protoplast yield (Zhang *et al*., 2011). Short incubation time results in incomplete digestion of protoplast, while long incubation time results in over-digestion of protoplast and thus, affecting the viability of isolated protoplasts. Therefore, optimal enzyme incubation period is critical in isolating viable protoplasts. Enzyme incubation period might vary between plant species with different cell wall composition and concentration of enzyme cocktail used to isolate protoplasts (Tee *et al*., 2010).

Similar finding was reported by Geetha *et al*. (2000), where 24 h was found to be optimal incubation time to produce maximum yield of protoplasts in cardamom suspension culture. However, prolonged incubation period to 48 h decreased the protoplasts yield in the present study. This was in agreement with the study carried out by Mazarei *et al*. (2011). The authors reported that prolonged incubation time did not increase the protoplast yield in *Panicum virgatum*. Over-digestion might cause the protoplasts to break, dysfunction, increased membrane instability and sensitivity of enzymatic solution (Zhang *et al*., 2011; Silva Jr. *et al*., 2012).

4.3 Viability test

Isolated protoplasts were spherical in shape and occurred as single cells after cell wall digestion (Figure 4.9A). Isolated protoplasts from *B. rotunda* suspension culture were stained with fluorescein diacetate (FDA) to test for protoplast viability. From the population, 54.93 ± 0.52 % of the isolated protoplasts (Appendix B, Table 6) were viable (Figures 4.9B & C). The viable protoplasts exhibited green fluorescence when observed under fluorescent microscope with blue light excitation, whereas non-viable protoplasts remained colourless. The fluorescence resulted from intracellular hydrolysis of FDA with fluorescein that passed through cell membranes and accumulated inside the cell.

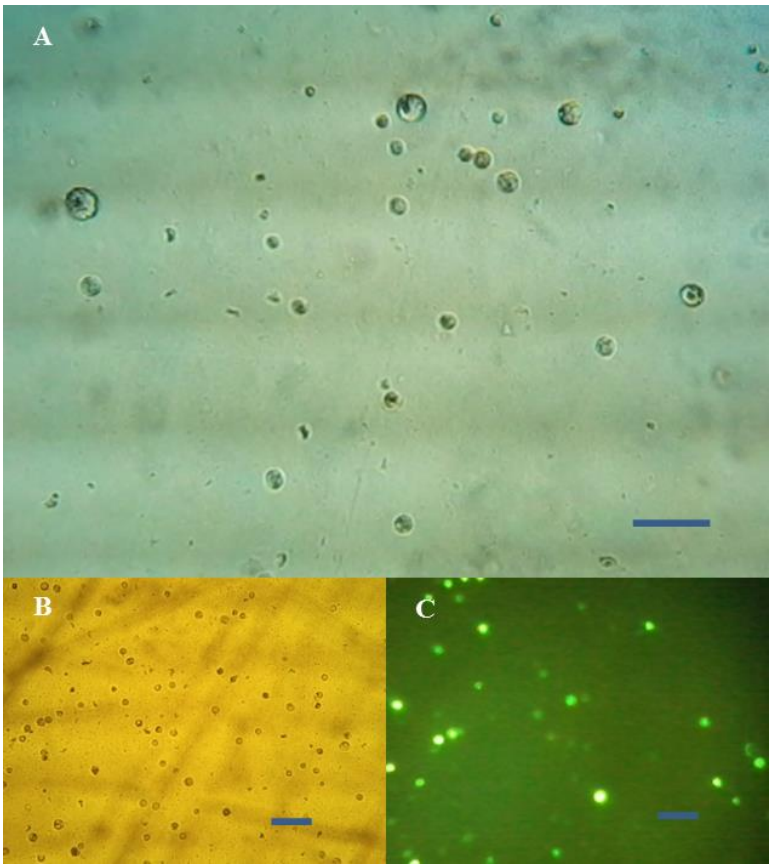

Figure 4.9: Protoplasts isolated from *B. rotunda* suspension cell culture under observation using an inverted microscope. **A:** isolated protoplast, bar indicates 125 µm. **B:** protoplasts stained with FDA viewed under normal light, bar indicates 125 µm. **C:** viable protoplasts appeared green fluorescent under blue light, bar indicates 125 µm.

4.4 Recovery of protoplast

Success in isolating protoplasts is depended on high percentage of viable protoplasts obtained. Viable protoplasts are able to recover cell wall and subsequently undergo cell division to form multi-celled callus and subsequently form plantlets. In this study, liquid medium was used to culture protoplasts in controlled conditions.

Plating density plays a key role in protoplasts culture regardless of any culture techniques used (Aziz *et al*., 2006; Al-Khayri, 2012). Previous study showed that plating density range between 0.5-10 $\times 10^5$ protoplasts per mL was effective to recover protoplast in many plant species (Davey *et al.*, 2005). In this study, 1-5 $\times 10^5$ protoplasts per mL was used for culture in MS medium (Appendix A, Table 5). Recovery of protoplasts is highly dependent on the plating density as it might affect 'cell-to-cell' communication between protoplasts (Ochatt and Power, 1992). Inappropriate plating density hindered cell division in protoplast culture due to nutrition depletion or lack of growth stimulus factors (Davey *et al.*, 2005; Aziz *et al*., 2006; Al-Khayri, 2012). It was reported that plating density of 1 $\times 10^4$ cells per mL resulted in maximum plating efficiency of 14.6 % in date palm protoplasts (Al-Khayri, 2012).

The formation of cell wall was confirmed by calcofluor white M2R staining. White fluorescent was observed after 24 h (Figures 4.10C & D) on viable protoplasts with cell wall formation. Protoplasts without cell wall formation did not fluorescent under UV microscope after staining with calcofluor white M2R. Cultured protoplasts started to form new cell wall after 24 h and complete new cell wall formation was seen after 2 days of culture (Figure 4.10D).

In this study, protoplast cultures were placed in the dark throughout the culture period as high intensity of light inhibited protoplast growth especially at the beginning of cultivation (Compton *et al.,* 2000; Chawla, 2002). *B. rotunda* protoplasts started to develop to 2-cell stage after five days (Figure 4.10A), followed by 4-cell stage at day 7 (Figure 4.10B). However, protoplast division is not synchronous in this study. The growth of protoplasts might be affected by repeated exposure of cultures to light source at the beginning of culture. The first cell division was also observed after 4-5 days of culture in *Musa paradisiacal* protoplast and subsequently the second cell division was recorded after 7 days of culture (Dai *et al*., 2010).

After 4 weeks, about 7.61 ± 1.65 % (Appendix B, Table 7) cultured protoplasts divided to form micro-colonies. The percentage of micro-colonies formation was higher compared to pear (Ochatt and Power, 1988), avocado (Witjaksono *et al.,* 1998) and *Mangifera indica* L. (Rezazadeh *et al.,* 2011). These micro-colonies were transferred to solid MS medium containing 0.5 mg/L BAP for callus initiation. Approximately 0.05 % micro-colonies formed callus after 5 weeks of culture (Figure 4.10E).

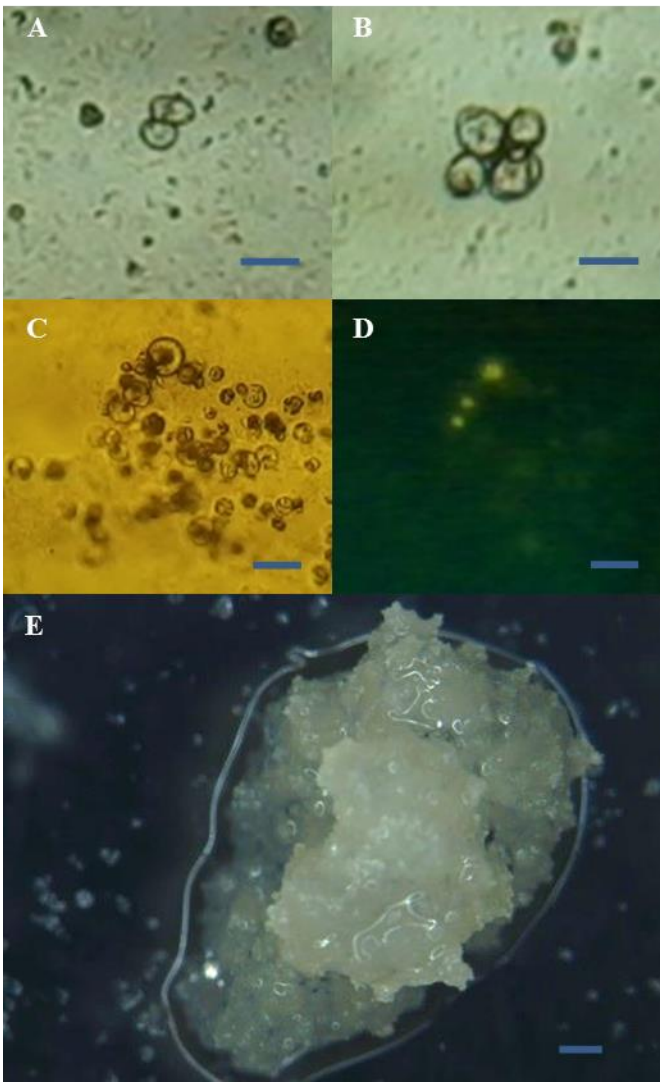

Figure 4.10: Recovery of the protoplasts at different developmental stages. **A:** 2-cell stage for first 5 days, bar indicates 100 µm. **B:** 4-cell stage at day 7, bar indicates 100 µm. **C:** protoplasts stained with calcofluor white M2R after 24 h of culturing viewed under normal light, bar indicates 500 µm. **D:** cell wall appeared white fluorescent under UV light, bar indicates 500 µm. **E:** friable callus derived from protoplast, bar indicates 1 mm.

In this study, both solid and liquid culture methods were used. However, protoplast division was initiated only in liquid medium. Different protoplast culture methods have been tested since 1980's. Liquid and solid MS media were initially used to culture protoplasts, however, some species were amenable to culture using liquid media while some were not. Other improvisation on culture methods include semi-solid culture, nurse culture and nurse cultures with a feeder layer. It was reported co-cultivation protoplast with a feeder layer was also able to improve cell division efficiency (Veera *et al*., 2009; Sheng *et al*., 2011).

5.0 Conclusion

In conclusion, this project has established a successful protocol for suspension culture, protoplast isolation and culture followed by callus initiation. An optimal cell growth of *B. rotunda* cell suspension culture has been obtained in PGR-free MS medium containing 3 % sucrose. A maximum protoplasts yield was obtained after 24 h of incubation period in enzyme cocktail of 1 % cellulase and 0.25 % macerozyme. Protoplast formed complete cell wall after 48 h and started to divide after 5 days and the cultures eventually formed callus. This study provides a platform for further research which can be applied in crop improvement programmes and secondary metabolite production mainly in protoplasts fusion and genetic transformation technologies. Further improvement on protoplast isolation protocol is still needed by using other types of enzymes, and also different culture media and methods.

6.0 References

Abdelwahab, S.I., Mohan, S., Abdulla, M.A., Sukari, M.A., Abdul, A.B., Elhassan Taha, M.M., Syam, S., Ahmad, S. and Lee, K.H. (2011). The methanolic extract of *Boesenbergia rotunda* (L.) Mansf. and its major compound pinostrobin induces anti-ulcerogenic property*in vivo*: Possible involvement of indirect antioxidant action. Journal of Ethnopharmacology, 137(2), 963-970.

Abdulla, M., Ali, H., Ahmed, K., Noor, S. and Ismail, S. (2009). Evaluation of the anti-ulcer activities of *Morus Alba* extracts in experimentally-induced gastric ulcer in rats. *Biomedical Research, 20,* 01-2009.

Abdullah, M.A., Ali, A.M., Marziah, M., Lajis, N.H. and Ariff, A.B. (1998). Establishment of cell suspension cultures of *Morinda elliptica* for the production of anthraquinones. *Plant Cell, Tissue and Organ Culture, 54*(3), 173-182.

Aitken-Christie, J. (1991). Automation. *In: Debergh PC & Zimmerman RH (eds) Micropropagation: Technology and Application. Kluwer Academic Publishers, Dordrecht,* 363-388.

Al-Khayri J.M. (2012). Determination of the date palm cell suspension growth curve, optimum plating efficiency, and influence of liquid medium on somatic embryogenesis. *Emirates Journal of Food and Agriculture, 24(5),* 444-455.

Aziz, Z.A., Davey, M.R., Lowe, K.C. and Power, J.B. (2006). Isolation and culture of protoplasts from the medicinal plant *Centella asiatica*. *Rev. Bras. Pl. Med. 8*,105-109.

Basaran, P. and Cerezo, E.R. (2008). Plant molecular farming: opportunities and challenges. *Crit rev biotechnology, 28,* 153-172.

Bhamarapravati, S., Juthapruth, S., Mahachai, W. and Mahady, G. (2006). Antibacterial activity of *Boesenbergia rotunda (L.) Mansf.* and *Myristica fragrans Houtt.* against *Helicobacter pylori*. *Songklanakarin J Sci Technol., 28*, 157-163.

Bhamarapravati, S., Mahady, G.B. and Pendland, S.L. (2003). In vitro susceptibility of *Helicobacter pylori* to extracts from the Thai medicinal plant *Boesenbergia rotunda* and Pinostrobin. *Proceedings of the 3rd World Congress on Medicinal and Aromatic Plants for Human Welfare, Chiang Mai Thailand*, 521.

Bhojwani, S.S. and Razdan, M.K. (1983). Plant tissue culture: theory and practise. *Amsterdam, Elsevier*, 237-286.

Bohm, H., Anthony, P., Davey, M.R., Briarty, L.G., Power, J.B., Lowe, K.C., Benes, E. and Groschl, M. (2000). Viability of plant cell suspensions exposed to homogeneous ultrasonic fields of different energy density and wave type. Plant Science Division, School of Biological Sciences, University of Nottingham, UK.

Boivin, E.B., Lepage, E., Matton, D.P., Crescenzo, G. and Jolicoeur, M. (2010). Transient expression of antibodies in suspension plant cell suspension cultures is enhanced when co-transformed with the tomato bushy stunt virus p19 viral suppressor of gene silencing. *Biotechnology progress, 26(6)*, 1534-1543.

Boonjaraspinyo, S., Boonmars, T., Aromdee, C. and Kaewsamut, B. (2010). Effect of fingerroot on reducing inflammatory cells in hamster infected with opisthorchis viverrini and N-nitrosodimethylamine administration. *Parasitol. Res., 106(6),* 1485-1489.

Burgess, J. and Fleming, E.N. (1974). Ultrastructural observations of cell wall regeneration around isolated tobacco protoplasts. *J. Cell Sci., 14*, 439-49.

Cai, Z.Z., Kastell, A., Knorr, D. and Smetanska, I. (2011). Exudation: an expanding technique for continuous production and release of secondary metabolites from plant cell suspension and hairy root cultures. *Plant cell reports, 31(3),* 461-477.

Castellar, A., Gagliardi, F. and Mansur, E. (2011). In vitro propagation and establishment of callus and cell suspension cultures of *Petiveria alliacea* L., a valuable medicinal plant. *Journal of medicinal plants research, 5(7),* 1113-1120.

Chan, E.W.C., Lim, Y.Y., Wong, L.F., Lianto, F.S., Wong, S.K., Lim, K.K., et al. (2008). Antioxidant and tyrosinase inhibition properties of leaves and rhizomes of ginger species. *Food Chem, 109,* 477-483.

Chawla, H.S. (2002). Introduction to plant biotechnology s edition. *Science Publisher, USA,* 91.

Chen, L.C-M, Craigie, J.S. and Xie, Z.K. (1994). Protoplast production from *Porphyra linearis* a simplified agarase procedure capable of commercial application. *Journal of Applied Phycology*, 635-639.

Chen, S., Tao, L., Zeng, L., Vega-Sanchez, M.E., Umemura, K. and Wang, G.L. (2006). A highly efficient transient protoplast system for analyzing defence gene expression and protein-protein interactions in rice. *Molecular Plant Pathology, 7(5),* 417–427.

Ching, A.Y.L., Wah, T.S., Sukari, M.A., Lian, G.E.C., Rahmani, M. and Khalid, K.A. (2007). Characterization of flavonoid derivatives from *Boesenbergia rotunda (L.). Malays J Anal Sci., 11,* 154-159.

Chomchalow, N., Bansiddhi, J. and Chantrasmi, V. (2006). Amazing Thai medicinal plants. *Royal Flora Ractchphruek 2006, Horticultural Research Institute and Horticultural Science Society of Thailand, Bangkok,* 10-11.

Cocking, E.C. (1960). A method for the isolation of plant protoplasts and vacuoles. *Nature, 187,* 962-963.

Compton, M.E., Saunders, J.A. and Veilleux, R.E. (2000). Use of protoplasts for plant improvement. *CRC Press LLC, 26,* 249-261.

Dai, X.M., Xiao, W., Huang, X., Zhao, J.T., Chen, Y.F. and Huang, X.L. (2010). Plant regeneration from embryogenic cell suspensions and protoplasts of dessert banana cv. 'Da Jiao' (*Musa paradisiacal* ABB Linn.) via somatic embryogenesis. *In vitro Cellular and Development Biology, 46*, 403-410.

Davey, M.R., Anthony, P., Power, J.B. and Lowe, K.C. (2005). Plant protoplasts: status and biotechnological perspectives. *Biotechnol. Adv., 23*, 131-171.

Davey, M.R., Marchant, R. and Power, J.B. (2003). Protoplasts of grain and forage legumes: their exploitation in genetic manipulation, physiological investigations and plant-pathogen interactions. *In: Jaiwal PK, Singh RP, editors. Improvement strategies for leguminosae biotechnology. Dordrecht, The Netherlands7 Kluwer Academic Publishers,* 133 -53.

Davey, M.R., Power, J.B. and Lowe, K.C. (2000). Plant protoplasts. *In: Spier RE, editor. Encyclopaedia of cell technology. New York, USA7 John Wiley and Sons,* 1034- 42.

Dědičová, B. (1995). Rastlinné protoplasty. *Biol. Listy, 60*, 241-257.

Erikson, T.R. (1986). Protoplast isolation and culture. *In: FOWKE L.C., CONSTABEL F. (eds.). Plant Protoplast. Florida, CRC Press Inc., Boca Raton, s printing,* 1-20.

Frimayanti, N., Zain, S.M., Lee, V.S., Wahab, H.A., Yusof, R. and Rahman, N.A. (2011/2012). Fragment-based molecular design of new competitive dengue Den2 Ns2b/Ns3 inhibitors from the components of fingerroot *(Boesenbergia rotunda). In Silico Biology, 11,* 29-37.

Gamborg, O.L., Miller, R.A. and Ojima, K. (1968). Nutrient requirements of suspension cultures of soybean root cells. *Exp. Cell Res., 50*, 151–8.

Geetha, S.P., Babu, K.N., Rema, J. Ravindran, P.N. and Peter, K.V. (2000). Isolation of protoplasts from cardamom (*Elettaria cardamomum* Maton.) and ginger (*Zingiber officinale* Rosc.). J Spices Aromatic Crops, 9(1), 23-30.

Godo, T., Komori, M., Nakaoki, E., Yukawa, T. and Miyoshi, K. (2010). Germination of mature seeds of *Calanthe tricarinata* Lindl., an endangered terrestrial orchid, by asymbiotic culture *in vitro*. *In Vitro Cellular Development Biology Plant, 46*, 323-328.

Grosser, J.W. and Gmitter Jr, F.G. (2011). Protocol fusion for production of tetraploid and triploid: application for scion and rootstock breeding in citrus. *Plant Cell Tissue and Organ Culture, 104*, 343-357.

Guo, Y.H., Bai, J.H. and Zhang, Z.H. (2007). Plant regeneration from embryogenic suspension-derived protoplasts of ginger. *Plant Cell Tissue Organ Culture, 89*, 151-157.

Hassanein, A., Hamama, L., Loridon, K. and Dorion, N. (2009). Direct gene transfer study and transgenic plant regeneration after electroporation into mesophyll protoplasts of *Pelargonium x hortorum*, 'Panaché Sud'. *Plant cell reports, 28(10)*, 1521-1530.

Ho, H.Y., Liang, K.Y., Lin, W.C., Kitanaka, S. and Wu, J.B. (2010). Regulation and improvement of triterpene formation in plant cultured cells of *Eriobotrya japonica* Lindl. *Journal of Bioscience and Bioengneering, 110*(5), 588-592.

Huang, T.K. and McDonald, K.A. (2012). Molecular farming using bioreactor-based plant cell suspension cultures for recombinant protein production. *Molecular farming in plants: recent advances and future prospects*, 37-67.

Iantcheva, A., Vlahova, M., Atanassov, A., Duque, A.S., Araújo, S., Dos Santos, D.F. and Fevereiro, P. (2006). Cell suspension cultures. *Medicago Truncatula Handbook,* 1-12.

Isa, N.M., Abdelwahab, S.I., Mohan, S., Abdul, A.B., Sukari, M.A., Taha, M.M.E., Syam, S., Narrima, P., Cheah, S.Ch., Ahmad, S. and Mustafa. M.R. (2012). *In vitro* anti-inflammatory, cytotoxic and antioxidant activities of Boesenbergin A, A Chalcone isolated from *Boesenbergia rotunda* (L.) (fingerroot). *Braz J Med Biol Res, 45(6),* 524-530.

Jimenez, V.M., Guevara, E., Herrera, J. and Bangerth, F. (2005). Evolution of endogenous hormone concentration in embryogenic cultures of carrot during early expression of somatic embryogenesis. *Plant Cell Reports, 23*(8), 567-572.

Jude, W.G. and Fred, G.G. (2011). Protoplast fusion for production of tetraploids and triploids: applications for scion and rootstock breeding in citrus. Plant Cell Tiss Organ Cult, 104, 343-357.

Kao, K.N. and Michayluk, M.R. (1980). Plant regeneration from mesophyll protoplasts of alfaalfa. *Z. Pflanzenphysiol., 96,* 135-141.

Kativat, C., Poolsawat, O. and Tantasawat, P.A. (2012). Optimisation of factors for efficient isolation of protoplasts in sunflower (*Helianthus anuus* L.). *Australian journal of crop science, 6(6),* 1004-1010.

Kirana, C., Jones, G.P., Record, I.R. and McIntosh, G.H. (2007). Anticancer properties of panduratin A isolated from *B. pandurata* (Zingiberaceae). *J. Nat. Med., 61,* 131-137.

Koch, C, Radel, S., Groschl, M., Benes, E. and Coakley, W.T. (2007). Effects of ultrasonic plane wave fields on yeast cultures. *3rd Congress of the Alps Adria Acoustics Association*, 27–28 September 2007.

Kothari, S.L., Joshi, A., Kachhwaha, S. and Ochoa-Alejo, N. (2010). Chilli peppers: a review on tissue culture and transgenesis. *Biotechnology advances, 28(1),* 35-48.

Landgren, C.R. (1978). Preparation of protoplasts of plant cell. *Methods in Cell Biology, 20,* 159-168.

Lau, O.S. and Sun, S.S.M. (2008). Plant seeds as bioreactors for recombinant protein production. *Elsevier: Biotechnology advances, 27(6),* 1015-1022.

Lee, E.J., Mobin, M., Hahn, J.E. and Paek, K.Y. (2006). Effects of sucrose, inoculum density, auxin, and aeration volume on cell growth of *Gymnema sylvestre*. *Journal of Plant Biology, 49*(6), 427-431.

Leguay, J.J. and Guern J. (1975). Quantitative effects of 2,4-dichlorophenoxyacetic acid on growth of suspension-cultured *Acer pseudoplatanus* cells. *Plant Physiol, 56,* 356-359.

Ling, J.J., Mohamed, M., Rahmat, A. and Abu Bakar, M.F. (2010). Phytochemicals, antioxidant properties and anticancer investigations of the different parts of several gingers species (*Boesenbergia rotunda*, *Boesenbergia pulchella* var attenuata and *Boesenbergia armeniaca*). *Journal of Medicinal Plants Research, 4(1),* 27-32

Lord, C. and Gunawardena, A. (2010). Isolation of leaf protoplasts from the submerged aquatic monocot *Aponogeton madagascariensis*. *Americes journal of plant science biotechnology, 4,* 6-11.

Mastuti, R., Miyake, H., Taniguchi, T. and Takeoka, Y. (2003). Isolation and culture of *Celosia cristata* L. cell suspension protoplasts. *Journal of Biological Researchers, 9(1),* 1-6.

Mazarei, M., Al-Ahmad, H., Rudis, M.R., Joyce, B.L. and Stewart Jr, C.N. (2011). Switchgrass (Panicum virgatum L.) cell suspension cultures: establishment, characterization, and application. *Elsevier Science: Plant Science, 181*, 712-715.

Miller, M.W., Miller, D.L. and Brayman, A.A. (1996). A review of in vitro bioeffects of inertial ultrasonic cavitation from a mechanistic perspective. *Elsevier Science, Ultrasound in Medicine and Biology, 22(9),* 1131–1154.

Morikawa, T., Funakoshi, K., Ninomiya, K., Yasuda, D., Miyagawa, K., Matsuda, H. and Yoshikawa, M. (2008). Medicinal foodstuffs. 34. Structures of new prenylchalcones and prenylflavanones with TNF-alpha and aminopeptidase N inhibitor activities from *Boesenbergia rotunda* (Tokyo). *Chem. Pharm. Bull., 56,* 956-962.

Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol Plant, 15*, 473-97.

Mustafa, N.R., Winter, W., Iren, F. and Verpoorte, R. (2011). Initiation, growth and cryopreservation of plant cell suspension cultures. *Nature protocols, 6(6),* 715-742.

Nagata, T. and Takebe, I. (1971). Plating of isolated tobacco mesophyll protoplasts on agar medium. *Planta, 99,* 12-20.

Ochatt, S.J. and Power, J.B. (1988). Plant regeneration from mesophyll protoplasts of Williams Bon Chretien (Syn Bartlett) pear (*Pyrus commonis* L.). *Plant Cell Rep., 7,* 587-589.

Ochatt, S.J. and Power, J.B. (1992). Plant regeneration from cultured protoplasts of higher plants. In: M.W. Fowler, G.S. Warren (eds) Plant biotechnology. Comprehensive Biotechnology s supplement. Oxford: Pergamon Press.

Panda, A.K., Mishra, S. and Bisaria, V.S. (1992). Alkaloid production by plant cell suspension cultures of *Holarrhena antidysenterica*: I. Effect of major nutrients. *Biotechnology and Bioengineering, 39*(10), 1043-1051.

Patil, R.S., Davey, M.R., Power, J.B. and Cocking, E.C. (2003). Development of long-term suspension cultures of wild tomato species, *Lycopersicon chilense* Dun. as regular source of protoplast: an efficient protoplast-to-plant system. *Indian Journal of Biotechnology, 2*, 504-511.

Phillips, G.C., Hubstenberger, J.F. and Hansen, E.E. (1995). Plant regeneration by organogenesis from callus and cell suspension cultures. *In: Gamborg OL, Phillips GC (eds), Plant Cell, Tissue and Organ Culture,* 67-78. Heidelberg: Springer and Verlag.

Pongchawee, K., Na-Nakhon, U., Lamseejan, S., Poompuang, S. and Phansiri, S. (2006). Factors affecting the protoplast isolation and culture of *Anubias nana* Engler. *Int J Bot.*, 2, 193-200.

Power, J.B. and Chapman, J.V. (1985). Isolation, culture and genetic manipulation of plant protoplasts. *In: Dixon, R.A. (ed.), Plant Cell Culture. A Practical Approach. Oxford, IRL Press,* 37-66.

Raikar, S.V., Braun, R.H., Bryant, C., Conner, A.J. and Christey, M.C. (2008). Efficient isolation, culture and regeneration of *Lotus corniculatus* protoplasts. *Plant biotechnology reports*, *2*, 171-177.

Rao, R.S. and Ravishankar, G.A. (2002). Plant cell cultures: Chemical factories of secondary metabolites. *Biotechnology Advances, 20*(2), 101-153.

Rehman, R.U., Stigliano, E., Lycett, G.W., Sticher, L., Sbano, F., Faraco, M., Dalessandro, G. and Di Sansebastiano, G.P. (2008). Tomato Rab11a characterization evidenced a difference between SYP121-dependent and SYP122-dependent exocytosis. *Plant Cell Physiology*, *49(5)*, 751–766.

Rezazadeh, R., Williams, R.R. and Harrison, D.K. (2011). Factors affecting mango (*Mangifera indica* L.) protoplast isolation and culture. *Scientia Horticulturae, 130,* 214-221.

Roest, S. and Gillisen, L.J.W. (1989). Plant regeneration from protoplasts: a literature review. *Acta Bot. Neer., 38,* 1–23.

Saralamp, P., Chuakul, W., Temsiririrkkul, R. and Clayton, T. (1996). Medicinal Plants in Thailand. *Amarin Printing and Publishing Public Co., Ltd., Bankok, 1,* 49.

Schlafer, O., Sievers, M., Klotzbucher, H. and Onyeche, T.I. (2000). Improvement of biological activity by low energy ultrasound assisted bioreactors. Elsevier: Ultrasonics, 38, 711–716.

Schenk, R.U. and Hildebrant, A.C. (1969). Production of protoplasts from plant cells in liquid culture using purified commercial cellulases. *Crop Sci. 9,* 629-631.

Sheen, J. (2001). Signal transduction in maize and Arabidopsis mesophyll protoplasts. *Plant Physiology, 127,* 1466–1475.

Science service. (2013). Fuchs Rosenthal Counting Chamber. Retrieved from http://scienceservices.de/media/pdf/ScienceServices_Fuchs_Rosenthal.pdf.

Sheng, X.G., Zhao, Z.Q., Yu, H.F., Wang, J.S., Zhang, X.H. and Gu, H.H. (2011). Protoplast isolation and plant regeneration of different double haploid lines of cauliflower (*Brassica oleracea* var. *botrytis*). *Plant Cell Tissue Culture Organ, 107,* 513-520.

Shih, S.M-H, Doran, P.M. 2008. Foreign protein production using plant cell and organ cultures: advantages and limitations. *Elsevier: Biotechnology advances, 27(6),* 1036-1042.

Shin, Y.K., Baque, M.A., Elghamedi, S., Lee, E.J. and Paek, K.Y. (2011). Effects of activated charcoal, plant growth regulators and ultrasonic pre-treatments on in vitro germination and protocorm formation of *Calanthe* hybrids. *Australian Journal of Crop Science, 5(5),* 582-588.

Shindo, K., Kato, M., Kinoshita, A., Kobayashi, A. and Koike, Y. (2006). Analysis of antioxidant activities contained in the *Boesenbergia pandurata* Schult. rhizome. *Biosci. Biotechnol. Biochem., 70(9),* 2281-2284.

Silva Jr., J.M., Paiva, R., Campos, A.C.A.L., Rodrigues, M., Carvalho, M.A.F. and Otoni, W.C. (2012). Protoplast production and isolation from *Etlingera elatior*. *Acta scientiarum: Agronomi, 34(1),* 45-50.

Sinha, A, Wetten, A.C., Caligari, P.D.S. (2003). Effect of biotic factors on the isolation of *Lupinus albus* protoplasts. *Aust J Bot, 51,* 103-9.

Sohn, J.H., Han, K.L., Lee, S.H. and Hwang, J.K. (2005). Protective effects of panduratin A against oxidative damage of tert-butylhydroperoxide in human HepG2 Cells. *Biological & Pharmaceutical Bulletin, 28,* 1083-1086.

Stevenson, P.C., Veitch, N.C. and Simmonds, M.S. (2007). Polyoxygenated cyclohexane derivatives and other constituent from *Kaempferia rotunda* L. *Phytochemistry, 68,* 1579-1586.

Sudwan, P., Saenphet, K., Aritajat, S. and Sitasuwan, N. (2007). Effects of *Boesenbergia rotunda* (L.) Mansf. on sexual behaviour of male rats. *Asian J Androl 2007, 9,* 849-855.

Sun, J., Chu, Y.F., Wu, X. and Liu, R.H. (2002). Antioxidant and antiproliferative activities of fruits. *J. Agric. Food Chem., 50*, 7449-7454.

Sun, Y.Q., Liu, S.M., Wang, Y., Jones, B.J., Wang, H.Z. and Zhu, S.J. (2011). An interspecific somatic hybrid between upland cotton (*G. hirsutum* L. cv. ZDM-3) and wild diploid cotton (*G. klotzschianum* A.). *Plant cell tissue and organ culture, 106*, 425-433.

Tan, E.C., Lee, Y.K., Chee, C.F., Heh, C.H., Wong S.M., Thio, C.L.P., Foo, G.T., Khalid, N., Abdul Rahman, N., Karsani, S.A., Othman, S., Othman, R. and Yusof, R. (2012a). *Boesenbergia rotunda*: From Ethnomedicine to Drug Discovery. *Evidence-Based Complementary and Alternative Medicine*, 25.

Tan, E.C., Karsani, S.A., Foo, G.T., Wong, S.M., Abdul Rahman, N., Khalid, N., Othman, S. and Yusof, R. (2012b). Proteomic analysis of cell suspension cultures of *Boesenbergia rotunda* induced by phenylalanine: identification of proteins involved in flavonoid and phenylpropanoid biosynthesis pathways. *Plant Cell Tissue and Organ Culture, 111*, 219-229.

Tan, S.K., Pippen, R., Yusof, R., Ibrahim, H., Rahman, N. and Khalid, N. (2005). Simple one-medium formulation regeneration of fingerroot [*Boesenbergia rotunda* (L.) mansf. Kulturpfl.] via somatic embryogenesis. *In Vitro Cellular Development Biology, 41*, 757-761.

Tan, S.K., Pippen, R., Yusof, R., Ibrahim, H. and Khalid Rahman, N.A. (2006). Inhibitory activity of cylohexenyl chalcone derivatives and favanoids of fingerroot, *Boesenbergia rotunda* (l), towards dengue-2 virus NS3 protease. *Bioorg. Med. Chem. Lett 2006, 16*, 3337-3340.

Tee, C.S, Lee, P.S., Anna, L.P.K. and Mahmood, M. (2010). Optimisation of protoplast isolation protocols using in vitro leaves of *Dendrobium crumenatum* (pigeon orchid). *African Journal of Agricultural Research, 5(19)*, 2685-2693.

Tewes, A. et al. (1984). High yield isolation and recovery of protoplast from suspension cultures of tomato (*L. esculentum*). *Z Pflanzenphysiol, 113*, 141-150.

Tewtrakul, S., Subhadhirasakul, S. and Kummee, S. (2003a). HIV-1 protease inhibitory effects of medicinal plants used as self-medication by AIDS patients. *Songklanakarin J. Sci. Technol., 25*, 239-243.

Tewtrakul, S., Subhadhirasakul, S., Puripattanavong, J. and Panphadung, T. (2003b). HIV-1 protease inhibitory substances from the rhizomes of *Boesenbergia pandurata* Holtt. *Songklanakarin J. Sci. Technol., 25,* 503-508.

Trakoontivakorn, G., Nakahara, K., Shinmoto, H., Takenaka, M., Onishi- Kameyama, M., Ono, H., Yoshida, M., Nagata, T. and 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. Agr. Food. Chem., 49,* 3046-3050.

Tuchinda, P., Reutrakul, V., Claeson, P., Pongprayoon, U., Sematong, T., Santisuk, T., et al. (2002). Anti-inflammatory cyclohexenyl chalcone derivatives in *Boesenbergia pandurata. Phytochemistry 2002, 59,* 169-173.

Uchimiya, H. and Murashige, T. (1974). Evaluation of parameters in the isolation of viable protoplast from cultured tobacco cells. *Plant Physiology, 54,* 936-944.

Valluri, J.V. (2009). Bioreactor producton of secondary metabolites from cell cultures of periwinkle and sandalwood. *Methods in molecular biology, 547,* 325-335.

Veera, R.N., Gregory, D.N., Philip, J.D. and Trevor, W.S. (2009). Regeneration from leaf explants and protoplasts of *Brassica oleracea* var. *botrytis* (cauliflower). *Science Horticulture, 119,* 330-334.

Voravuthikunchai, S.P., Phongpaichit, S. and Subhadhirasakul, S. (2005). Evaluation of antibacterial activities of medicinal plants widely used among AIDS patients in Thailand. *43,* 701-706.

Wang, J., Sun, Y., Yan, S., Daud, M.K. and Zhu, S. (2008). High frequency plant regeneration from protoplast in cotton *via* somatic embryogenesis. *Biologia plantarium, 52(4),* 616-620.

Witjaksono, Litz, R.E. and Grosser, J.W. (1998). Isolation, culture and regeneration of avocado (*Persea Americana* Mill.) protoplasts. *Plant Cell Rep., 18*, 235-242.

Wu, C.H., Dewir, Y.H., Hahn, E.J. and Paek, K.Y. (2006). Optimization of culturing conditions for the production of biomass and phenolics from adventitious roots of *Echinacea angustifolia*. *Journal Plant Biology, 49*, 193-199.

Wu, J. and Lin, L. (2002). Elicitor-like effects of low-energy ultrasound on plant (*Panax ginseng*) cells: induction of plant defense responses and secondary metabolite production. *Applied Microbiology and Biotechnology, 59*, 51-57.

Xu, J.F., Ge, X.M. and Dolan, M.C. (2011). Towards high-yield production of pharmaceutical proteins with plant cell suspension cultures. *Elsevier: Biotechnology advances, 29(3)*, 278-299.

Yang, X.Y., Tu, L., Zhu, L.F., Fu, L., Min, L. and Zhang, X.L. (2008). Expression profile analysis of genes involved in cell wall regeneration during protoplast culture in cotton by suppression subtractive hybridization and macroarray. *Journal of Experimental Botany, 59(13),* 3661-3674.

Yotarlai, S., Chaisuksunt, V., Saenphet, K. and Sudwan, P. (2011). Effects of *Boesenbergia rotunda* juice on sperm qualities in male rats. *Journal of Medicinal Plants Research, 5(16),* 3861-3867.

Yusuf, N.A., Annuar, M.M.S. and Khalid, N. (2011a). Rapid micropropagation of *Boesenbergia rotunda* (L.) Mansf. Kulturpfl. (a valuable medicinal plant) from shoot bud explants. *African Journal of Biotechnology, 10*, 1194-1199.

Yusuf, N.A., Annuar, M.M.S. and Khalid, N. (2011b). Efficient propagation of an important medicinal plant *Boesenbergia rotunda* by shoot derived callus. *Journal of Medicinal Plants Research, 5(13)*, 2629-2636.

Yusuf, N.A. (2011c). Biomass and selected flavonoids production in cell suspension cultures of *Boesenbergia rotunda* (L.) MANSF. *Unpublished Dissertation and thesis*, University of Malaya.

Zhai, Z.Y., Thanwalee, S.N., and Vatamaniuk, O.K. (2009). Establishing rna interference as a reverse-genetic approach for gene functional analysis in protoplasts. *Plant Physiology, 149,* 642-652.

Zhang, J.B., Shen, W.T., Yan, P., Li, X.Y. and Zhou, P. (2011). Factors that influence the yield and viability of protoplasts from *Carica papaya* L. *African Journal of Biotechnology, 10(26),* 5137-5142.

Zhang, Y.H., Zhong, J.J. and Yu, J.T. (1996). Enhancement of ginseng saponin production in suspension cultures of *Panax notoginseng*: Manipulation of medium sucrose. *Journal of Biotechnology, 51*(1), 49-56.

Zhou, X., Wei, Y., Zhu, H., Wang, Z., Lin, J., Lu, L. and Tang, K. (2008). Protoplast formation, regeneration and transformation from the taxol-producing fungus *Ozonium sp. African Journal of Biotechnology, 7(12),* 2017-2024.

7.0 Appendices

7.1 Appendix A: Materials used in details

Table 1: Composition of Murashige and Skoog based media (MS basal salt).

Components	Concentration (mg/L)
Macronutrients	
$CaCl_2.2H_2O$	440.0
NH_4NO_3	1650.0
KNO_3	1900.0
KH_2PO_4	170.0
$MgSO_2.7H_2O$	370.0
Micronutrients	
KI	0.830
$CoCl_2.6H_2O$	0.025
H_3BO_3	6.200
$Na_2MoO_4.2H_2O$	0.250
$MnSO_4.4H_2O$	22.300
$CuSO_4.5H_2O$	0.025
$ZnSO_4.7H_2O$	8.600
FeEDTA	
FeSO4.7H2O	27.85
Na2EDTA.2H2O	37.25
Vitamins	
Glycine	2.0
Nicotinic Acid	0.5
Pyridoxine	0.5
Thiamine HCl	0.1

Table 2: Composition of liquid medium.

Components	**Concentration (mg/L)**
MS basal Salt	
Myo-inositol	100.00
Malt extract	150.00
Maltose	5000.00
Sucrose	30000.00
Biotin	1.00
BAP	1.00
NAA	1.00
2,4-D	2.00
glutamine	100.00

Table 3: Enzyme combination for protoplast isolation.

Cellulase / **Macerozyme**	1%	2%
0.25%	A	B
0.5%	C	D

Table 4: Composition of CPW13M and CPW21S.

CPW salt	**(Concentration (mg/L)**
KH_2PO_4	27.2
KNO_3	101.0
$CaCl_2.2H_2O$	1480.0
$MgSO_4.7H_2O$	246.0
KI	0.16
$CuSO_4.5H_2O$	0.025
For CPW13M	
Mannitol	130g
For CPW21S	
Sucrose	210g

Table 5: Composition of liquid protoplast culture.

Components	**Concentration (mg/L)**
MS basal salt	
Myo-inositol	100.00
Malt extract	150.00
Maltose	5000.00
Sucrose	30000.00
NAA	2.00
BAP	0.50
For MSP1 9M	
Mannitol	90000.00

7.2 Appendix B: Raw Data

Table 1: Suspension culture with 2,4-D treatment.

Day / Treatment	0	3	6	9	12	15	18	21	24	27
0 mg/L-1	1.00	1.10	1.50	1.70	2.00	2.60	3.30	3.70	3.60	3.90
0 mg/L-2	1.00	1.00	1.00	1.40	1.50	2.00	2.40	2.60	2.60	2.80
0 mg/L-3	1.00	1.10	1.60	1.80	2.30	3.00	3.60	4.30	4.20	4.30
ave.	1.00	1.07	1.37	1.63	1.93	2.53	3.10	3.53	3.47	3.67
SD	0.00	0.06	0.32	0.21	0.40	0.50	0.62	0.86	0.81	0.78
SE	0.00	0.03	0.19	0.12	0.23	0.29	0.36	0.50	0.47	0.45
2 mg/L-1	1.00	1.00	1.20	1.50	1.60	1.60	1.90	1.90	2.00	1.90
2 mg/L-2	1.00	1.10	1.50	1.50	1.50	1.70	1.90	1.90	2.10	2.80
2 mg/L-3	1.00	1.10	1.60	1.70	1.70	1.90	2.10	2.10	2.30	2.30
ave.	1.00	1.07	1.43	1.57	1.60	1.73	1.97	1.97	2.13	2.33
SD	0.00	0.06	0.21	0.12	0.10	0.15	0.12	0.12	0.15	0.45
SE	0.00	0.03	0.12	0.07	0.06	0.09	0.07	0.07	0.09	0.26
4 mg/L-1	1.00	1.00	1.00	1.20	1.70	1.90	2.20	2.30	2.20	2.50
4 mg/L-2	1.00	1.00	1.00	1.10	1.10	1.10	1.10	1.10	1.00	1.10
4 mg/L-3	1.00	1.10	1.50	1.60	1.80	1.80	2.20	2.40	2.40	2.50
ave.	1.00	1.03	1.17	1.30	1.53	1.60	1.83	1.93	1.87	2.03
SD	0.00	0.06	0.29	0.26	0.38	0.44	0.64	0.72	0.76	0.81
SE	0.00	0.03	0.17	0.15	0.22	0.25	0.37	0.42	0.44	0.47
8 mg/L-1	1.00	1.10	1.10	1.60	1.80	2.00	2.20	2.50	2.50	2.50
8 mg/L-2	1.00	1.00	1.10	1.50	1.60	1.70	1.80	2.00	2.10	2.30
8 mg/L-3	1.00	1.00	1.30	1.60	1.70	1.80	1.90	2.00	2.10	2.10
ave.	1.00	1.03	1.17	1.57	1.70	1.83	1.97	2.17	2.23	2.30
SD	0.00	0.06	0.12	0.06	0.10	0.15	0.21	0.29	0.23	0.20
SE	0.00	0.03	0.07	0.03	0.06	0.09	0.12	0.17	0.13	0.12
16 mg/L-1	1.00	1.00	1.00	1.20	1.60	1.70	1.80	2.00	2.00	2.00
16 mg/L-2	1.00	1.00	1.00	1.00	1.20	1.50	1.70	1.70	1.60	1.80
16 mg/L-3	1.00	1.10	1.10	1.00	1.10	1.00	1.10	1.10	1.00	1.10
ave.	1.00	1.03	1.03	1.07	1.30	1.40	1.53	1.60	1.53	1.63
SD	0.00	0.06	0.06	0.12	0.26	0.36	0.38	0.46	0.50	0.47
SE	0.00	0.03	0.03	0.07	0.15	0.21	0.22	0.26	0.29	0.27

Table 2: Suspension culture with sonication treatment.

Day / Treatment	0	3	6	9	12	15	18	21	24	27
0 s – 1	1.00	1.00	1.20	1.50	1.60	1.60	1.90	1.90	2.00	1.90
0 s - 2	1.00	1.10	1.50	1.50	1.50	1.70	1.90	1.90	2.10	2.80
0 s - 3	1.00	1.10	1.60	1.70	1.70	1.90	2.10	2.10	2.30	2.30
ave.	1.00	1.07	1.43	1.57	1.60	1.73	1.97	1.97	2.13	2.33
SD	0.00	0.06	0.21	0.12	0.10	0.15	0.12	0.12	0.15	0.45
SE	0.00	0.03	0.12	0.07	0.06	0.09	0.07	0.07	0.09	0.26
30 s - 1	1.00	1.60	1.30	1.30	1.50	1.30	1.30	1.20	1.20	1.10
30 s - 2	1.00	1.40	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
30 s - 3	1.00	1.50	1.60	1.40	1.40	1.40	1.20	1.20	1.20	1.20
ave.	1.00	1.50	1.30	1.23	1.30	1.23	1.17	1.13	1.13	1.10
SD	0.00	0.10	0.30	0.21	0.26	0.21	0.15	0.12	0.12	0.10
SE	0.00	0.06	0.17	0.12	0.15	0.12	0.09	0.07	0.07	0.06
120 s - 1	1.00	1.50	1.50	1.30	1.00	1.00	1.00	1.00	1.00	1.00
120 s - 2	1.00	1.40	1.40	1.40	1.00	1.00	1.00	1.00	1.00	1.00
120 s - 3	1.00	1.20	1.30	1.20	1.00	1.00	1.00	1.00	1.00	1.00
ave.	1.00	1.37	1.40	1.30	1.00	1.00	1.00	1.00	1.00	1.00
SD	0.00	0.15	0.10	0.10	0.00	0.00	0.00	0.00	0.00	0.00
SE	0.00	0.09	0.06	0.06	0.00	0.00	0.00	0.00	0.00	0.00
300 s - 1	1.00	1.20	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
300 s - 2	1.00	1.20	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
300 s - 3	1.00	1.10	1.10	1.00	1.00	1.00	1.00	1.00	1.00	1.00
ave.	1.00	1.17	1.03	1.00	1.00	1.00	1.00	1.00	1.00	1.00
SD	0.00	0.06	0.06	0.00	0.00	0.00	0.00	0.00	0.00	0.00
SE	0.00	0.03	0.03	0.00	0.00	0.00	0.00	0.00	0.00	0.00
600 s - 1	1.00	1.60	1.50	1.40	1.30	1.20	1.00	1.00	1.00	1.00
600 s - 2	1.00	1.10	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
600 s - 3	1.00	1.50	1.50	1.30	1.20	1.00	1.00	1.00	1.00	1.00
ave.	1.00	1.40	1.33	1.23	1.17	1.07	1.00	1.00	1.00	1.00
SD	0.00	0.26	0.29	0.21	0.15	0.12	0.00	0.00	0.00	0.00
SE	0.00	0.15	0.17	0.12	0.09	0.07	0.00	0.00	0.00	0.00

Table 3: Suspension culture with sucrose treatment.

Day / Treatment	0	3	6	9	12	15	18	21	24	27
0.0 % - 1	1.00	1.50	2.00	3.10	3.50	3.60	4.00	3.70	4.10	3.80
0.0 % - 2	1.00	1.60	2.10	3.10	3.50	3.70	4.00	4.00	4.40	4.10
0.0 % - 3	1.00	1.60	2.00	3.30	3.40	3.40	3.90	4.00	4.10	3.70
ave.	1.00	1.57	2.03	3.17	3.47	3.57	3.97	3.90	4.20	3.87
SD	0.00	0.06	0.06	0.12	0.06	0.15	0.06	0.17	0.17	0.21
SE	0.00	0.03	0.03	0.07	0.03	0.09	0.03	0.10	0.10	0.12
1.5 % - 1	1.00	1.60	2.00	3.80	4.90	7.10	8.70	8.50	9.70	9.70
1.5 % - 2	1.00	1.40	2.10	3.50	4.40	6.60	8.10	8.10	9.00	8.00
1.5 % - 3	1.00	1.70	2.10	4.00	4.80	6.90	8.00	8.10	8.90	7.40
ave.	1.00	1.57	2.07	3.77	4.70	6.87	8.27	8.23	9.20	8.37
SD	0.00	0.15	0.06	0.25	0.26	0.25	0.38	0.23	0.44	1.19
SE	0.00	0.09	0.03	0.15	0.15	0.15	0.22	0.13	0.25	0.69
3.0 % - 1	1.00	1.60	2.00	3.60	4.60	6.60	8.10	10.00	12.00	12.90
3.0 % - 2	1.00	1.50	2.10	3.50	4.60	6.70	8.00	11.00	14.00	14.50
3.0 % - 3	1.00	1.90	2.10	3.40	4.60	6.40	7.80	9.70	12.10	12.50
ave.	1.00	1.67	2.07	3.50	4.60	6.57	7.97	10.23	12.70	13.30
SD	0.00	0.21	0.06	0.10	0.00	0.15	0.15	0.68	1.13	1.06
SE	0.00	0.12	0.03	0.06	0.00	0.09	0.09	0.39	0.65	0.61
4.5 % - 1	1.00	1.60	2.20	3.50	4.40	6.30	7.40	9.70	11.80	13.40
4.5 % - 2	1.00	1.50	2.00	3.10	4.10	5.40	6.70	8.50	11.00	12.60
4.5 % - 3	1.00	1.50	1.90	2.70	3.70	5.20	6.40	8.00	10.70	12.30
ave.	1.00	1.53	2.03	3.10	4.07	5.63	6.83	8.73	11.17	12.77
SD	0.00	0.06	0.15	0.40	0.35	0.59	0.51	0.87	0.57	0.57
SE	0.00	0.03	0.09	0.23	0.20	0.34	0.30	0.50	0.33	0.33
6.0 % - 1	1.00	1.60	1.90	3.00	4.30	5.10	6.30	7.00	9.00	9.90
6.0 % - 2	1.00	1.60	2.20	3.00	4.30	5.70	6.10	6.90	8.30	9.70
6.0 % - 3	1.00	1.30	2.10	3.00	4.40	5.30	6.30	8.00	9.80	10.30
ave.	1.00	1.50	2.07	3.00	4.33	5.37	6.23	7.30	9.03	9.97
SD	0.00	0.17	0.15	0.00	0.06	0.31	0.12	0.61	0.75	0.31
SE	0.00	0.10	0.09	0.00	0.03	0.18	0.07	0.35	0.43	0.18

Table 4: Enzyme combination optimisation (protoplast isolated per SCV).

EnzymeA (10^5)			ave.	EnzymeB (10^5)			ave.	EnzymeC (10^5)			ave.	EnzymeD (10^5)			ave.
1.65	1.65	1.88		0.90	0.60	1.13		0.68	1.43	0.98		2.48	2.33	2.85	
2.48	2.18	0.98		0.98	1.50	1.05		0.60	1.35	1.35		1.80	2.25	2.55	
2.25	2.18	1.43		0.90	0.60	1.65		0.53	1.13	0.83		1.73	2.18	1.95	
2.25	1.73	1.95		0.83	0.45	1.43		0.53	0.90	0.68		2.48	2.48	2.03	
2.33	2.33	1.20		0.90	1.05	0.75		0.68	1.05	0.83		1.50	2.25	2.18	
2.19	2.01	1.49	1.90	0.90	0.84	1.20	0.98	0.60	1.17	0.93	0.90	2.00	2.30	2.31	2.20
0.32	0.30	0.42	0.45	0.05	0.43	0.35	0.34	0.07	0.22	0.26	0.30	0.45	0.11	0.38	0.35
0.18	0.17	0.24	0.26	0.03	0.25	0.20	0.20	0.04	0.12	0.15	0.18	0.26	0.07	0.22	0.20
2.48	2.55	2.10		0.68	0.45	0.68		1.05	1.05	0.60		1.80	1.65	2.40	
2.55	1.58	2.33		0.98	0.53	0.60		0.98	0.75	0.45		1.58	1.65	2.10	
2.93	2.78	2.03		0.53	0.68	1.28		1.05	0.68	0.98		1.88	1.58	2.10	
2.10	2.33	2.63		0.98	0.75	0.38		0.90	0.60	0.98		1.95	1.95	2.18	
1.88	1.88	2.48		0.83	0.38	0.30		0.98	0.90	0.90		2.48	1.80	2.33	
2.39	2.22	2.31	2.31	0.80	0.56	0.65	0.67	0.99	0.80	0.78	0.86	1.94	1.73	2.22	1.96
0.41	0.49	0.25	0.37	0.20	0.16	0.38	0.27	0.06	0.18	0.24	0.19	0.33	0.15	0.14	0.30
0.24	0.28	0.14	0.22	0.11	0.09	0.22	0.15	0.04	0.10	0.14	0.11	0.19	0.09	0.08	0.17
1.43	1.28	1.20		1.13	0.68	0.83		1.13	0.38	0.83		2.25	1.88	2.70	
2.25	1.50	1.35		1.13	1.28	1.20		0.53	0.45	0.75		2.78	2.18	2.40	
1.50	1.20	1.80		1.35	0.90	1.43		0.45	0.53	0.68		2.40	2.63	2.25	
2.25	2.48	1.28		0.83	0.75	0.90		0.60	0.38	0.53		2.85	2.78	2.18	
2.03	2.03	1.73		0.75	1.58	0.90		0.60	0.30	0.38		2.40	1.95	2.78	
1.89	1.70	1.47	1.69	1.04	1.04	1.05	1.04	0.66	0.41	0.63	0.57	2.54	2.28	2.46	2.43
0.40	0.54	0.27	0.43	0.25	0.38	0.25	0.28	0.27	0.09	0.18	0.21	0.26	0.40	0.27	0.31
0.23	0.31	0.16	0.25	0.14	0.22	0.15	0.16	0.15	0.05	0.10	0.12	0.15	0.23	0.15	0.18

Table 5: Enzyme incubation time optimisation (protoplast isolated per SCV).

	5 h (10^5)			ave.	24 h (10^5)			ave.	48 h (10^5)			ave.
	0.30	0.38	0.60		1.65	1.65	1.88		0.08	0.30	0.38	
	0.30	0.38	0.38		2.48	2.18	0.98		0.23	0.68	0.45	
	0.60	0.75	0.30		2.25	2.18	1.43		0.60	0.38	0.30	
	0.68	0.38	0.45		2.25	1.73	1.95		0.23	0.23	0.15	
	0.75	0.45	0.23		2.33	2.33	1.20		0.38	0.30	0.30	
ave.	0.53	0.47	0.39	0.46	2.19	2.01	1.49	1.90	0.30	0.38	0.32	0.33
SD	0.21	0.16	0.14	0.16	0.32	0.30	0.42	0.43	0.20	0.18	0.11	0.14
SE	0.12	0.09	0.08	0.09	0.18	0.17	0.24	0.25	0.11	0.10	0.06	0.08
	0.83	0.53	0.83		2.48	2.55	2.10		0.23	0.23	0.23	
	0.45	0.45	0.38		2.55	1.58	2.33		0.30	0.75	0.53	
	0.60	0.75	0.68		2.93	2.78	2.03		0.15	0.23	0.15	
	0.45	0.45	0.45		2.10	2.33	2.63		0.38	0.30	0.30	
	0.75	0.60	0.53		1.88	1.88	2.48		0.75	0.23	0.45	
ave.	0.62	0.56	0.57	0.58	2.39	2.22	2.31	2.31	0.36	0.35	0.33	0.35
SD	0.17	0.13	0.18	0.14	0.41	0.49	0.25	0.34	0.23	0.23	0.16	0.18
SE	0.10	0.07	0.10	0.08	0.24	0.28	0.14	0.20	0.13	0.13	0.09	0.10
	0.53	0.45	0.30		1.43	1.28	1.20		0.08	0.53	0.23	
	0.30	0.30	0.23		2.25	1.50	1.35		0.53	0.45	0.45	
	0.15	0.23	0.38		1.50	1.20	1.80		0.23	0.23	0.38	
	0.38	0.38	0.23		2.25	2.48	1.28		0.38	0.30	0.38	
	0.45	0.38	0.23		2.03	2.03	1.73		0.53	0.38	0.75	
ave.	0.36	0.35	0.27	0.33	1.89	1.70	1.47	1.69	0.35	0.38	0.44	0.39
SD	0.14	0.09	0.07	0.10	0.40	0.54	0.27	0.39	0.20	0.12	0.19	0.15
SE	0.08	0.05	0.04	0.06	0.23	0.31	0.16	0.23	0.11	0.07	0.11	0.09

Table 6: Viability of isolated protoplasts.

protoplast	Rep. 1	Rep. 2	Rep. 3	Ave.
total cell	204.00	203.00	212.00	206.33
viable cell	110.00	112.00	118.00	113.33

Table 7: Recovered protoplast during protoplast culture.

protoplast	Rep. 1	Rep. 2	Rep.3	Rep.4	Ave.
total cell	71	97	147	79	7.5
divided cell	4	5	12	9	98.5

7.3 Appendix C: Statistical Analysis

Table 1: Test of homogeneity of variances for different enzyme combination.

Levene Statistic	df1	df2	Sig.
1.484	3	32	.237

Table 2: ANOVA test for difference enzyme combination.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	6.330E10	3	2.110E10	66.396	.000
Within Groups	1.017E10	32	3.178E8		
Total	7.347E10	35			

Table 3: Homogenous subset for difference enzyme combination.

			Subset for alpha = 0.05	
	enzyme	N	1	2
Duncan[a]	3	9	5.1556E4	
	2	9	5.9667E4	
	1	9		1.3078E5
	4	9		1.4633E5
	Sig.		.342	.073

Table 4: Test of homogeneity of variances for difference enzyme incubation period.

Levene Statistic	df1	df2	Sig.
16.762	2	24	.000

Table 5: ANOVA test for difference enzyme incubation period.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	6.489E10	2	3.245E10	160.308	.000
Within Groups	4.858E9	24	2.024E8		
Total	6.975E10	26			

Table 6: Homogenous subset for difference enzyme incubation period.

			Subset for alpha = 0.05	
	Incubationhrs	N	1	2
Duncan[a]	3	9	2.3556E4	
	1	9	3.0333E4	
	2	9		1.3078E5
	Sig.		.322	1.000

Table 7: Test of homogeneity of variances for effects of different concentrations of 2,4-D on cell suspension growth rate from day 6 till day 18.

Levene Statistic	df1	df2	Sig.
2.638	4	10	.097

Table 8: ANOVA test for effects of different concentrations of 2,4-D on cell suspension growth rate from day 6 till day 18.

VAR00002					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.003	4	.001	2.231	.138
Within Groups	.004	10	.000		
Total	.007	14			

Table 9: Homogenous subset for effects of different concentrations of 2,4-D on cell suspension growth rate from day 6 till day 18.

	VAR00001	N	Subset for alpha = 0.05	
			1	2
Duncan[a]	2	3	.026900	
	5	3	.031067	
	3	3	.035167	.035167
	4	3	.043467	.043467
	1	3		.068767
	Sig.		.350	.069

Table 10: Test of homogeneity of variances for effect of various sonication times on cell suspension growth rate from day 6 till day 18.

Levene Statistic	df1	df2	Sig.
3.839	4	10	.038

Table 11: ANOVA test for effects of various sonication times on cell suspension growth rate from day 6 till day 18.

VAR00002					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.006	4	.001	9.506	.002
Within Groups	.001	10	.000		
Total	.007	14			

Table 12: Homogenous subset for effects of various sonication times on cell suspension growth rate from day 6 till day 18.

	VAR00001	N	Subset for alpha = 0.05		
			1	2	3
Duncan[a]	3	3	-.027900		
	5	3	-.022533	-.022533	
	2	3	-.008000	-.008000	
	4	3		-.002633	
	1	3			.026900
	Sig.		.082	.082	1.000

Table 13: Test of homogeneity of variances for effects of different concentrations of sucrose on cell suspension growth rate from day 6 till day 18.

Levene Statistic	df1	df2	Sig.
2.760	4	10	.088

Table 14: ANOVA test for effects of different concentrations of sucrose on cell suspension growth rate from day 6 till day 18.

VAR00002					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.007	4	.002	78.031	.000
Within Groups	.000	10	.000		
Total	.007	14			

Table 15: Homogenous subset for effects of different concentrations of sucrose on cell suspension growth rate from day 6 till day 18.

	VAR00001	N	Subset for alpha = 0.05			
			1	2	3	4
Duncan[a]	1	3	.055733			
	5	3		.092167		
	4	3			.101000	
	3	3				.112467
	2	3				.115500
	Sig.		1.000	1.000	1.000	.449