EVALUATION OF SHAKE FLASK AND BIOREACTOR SYSTEMS ON CELL GROWTH AND REGENERATION OF *MUSA ACUMINATA* CV. BERANGAN CELL SUSPENSION CULTURE

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ABSTRACT

In this study, a semi automated cell suspension culture protocol for *Musa acuminata* cv. 'Berangan' using bioreactor has been established. Flower clusters at the positions of 8 to 10 were the most responsive (70 %) towards embryogenesis. After eight months of culture, embryogenic calli generated from male inflorescences were transferred to liquid suspension medium. Embryogenic cell suspension culture was successfully established after two months of culture in liquid M2 medium. Cell suspension cultures showed optimum cell growth when cultured in the M2 modified liquid medium containing 2 % sucrose with an initial inoculum density of 2 % settled cell volume (SCV). The yield of cell suspension cultures was increased to 165 % and 210 %, respectively, when inoculated in 5 l balloon type bubble column bioreactors (BTBCBs) without pH control and with pH maintained at 5.7, over 14 days of culture. The results showed that the growth yield was 3-, 2.5- and 2.2- fold higher than initial culture (day 1) when cultured in pH-controlled BTBCB, non-pH-controlled BTBCB and shake flask respectively.In all growth vessels tested, catalase (CAT) activity was found to be correlated with hydrogen peroxide (H_2O_2) concentration, suggesting its effective scavenging activity, whereas no significant difference was observed for superoxide dismutase (SOD). More than 60 % of the embryos started to form shoots after 2 weeks of culture on M4 medium for all growth vessels tested. Abnormalities in plantlets were less than 20 % in all three types of culture vessels. Potentially, this protocol could be used to mass produce disease-free and high yielding banana as it provides a comparable cell growth rate and number of regenerants which phenotypically were identical to the donor plants compared to conventional shake flask cultures.

ABSTRAK

Dalam kajian ini, satu sistem semi-automatik penghasil sel ampaian bagi *Musa acuminata* cv. ‘Berangan’menggunakanbioreaktortelahberjayadiperolehi. Kelompok bunga jantan di posisi 8 hingga 10 menunjukkanresponse yang tertinggi (70 %) terhadap embryogenesis. Selepas lapan bulan, kalus embrio somatik yang dihasilkan daripada kelompok bunga jantan dipindahkan ke dalam media cecair. Ampaian sel embriogenik berjaya dihasilkan selepas dua bulan dikulturkan dalam media cecair M2. Ampaian sel menunjukkan pertumbuhan yang paling optima apabila dikultur di dalam media dimodifikasi M2 yang mengandungi 2 % (b/i) sukrosa dengan kepadatan inokulum permulaan (SCV) sebanyak 2 %. Jumlah penghasilan ampaian sel meningkat kepada 165 % dan 210 % selepas 14 hariapabiladikultur di dalam 5 liter bioreaktor jenis belon ruangan gelembung (BTBCB) tanpa kawalan pH dan dengan kawalan pH pada 5.7. Hasil pertumbuhan ampaian sel adalah 3-, 2.5- dan 2- kali ganda lebih banyak daripada kultur permulaan (hari pertama) apabila dikultur di dalam BTBCB dengan kawalan pH, BTBCB tanpa kawalan pH dan kelalang kon biasa. Pertumbuhan kultur sel diuji untuk semua bekas kultur dan aktiviti enzim katalase (CAT) didapati berkaitrapat dengan kepekatan hidrogen peroksida (H_2O_2), mencadangkan aktiviti memerangkap adalah berkesan. Manakala tiada perbezaan ketara diperhatikan bagi enzim superoksida dismutase (SOD). Lebih daripada 60 % embrio mula menjana pucuk selepas dua minggu dikultur di dalam media M4. Keabnormalan pucuk adalah kurang daripada 20 % untuk semua bekas kultur yang diuji. Protokol ini berpotensi untuk menghasilkan tumbuhan pisang yang bebas daripada penyakit dan penghasilan tinggi kerana ia menunjukkan kadar pertumbuhan dan jumlah pucuk yang serupa dari segi fenotip dengan pokok induk setara dengan kultur ampaian sel yang konvensional.

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LIST OF SYMBOLS AND ABBREVIATIONS

%	Percentage
℃	Degree Celsius
2,4-D	2,4-dichlorophenoxyacetic acid
ANOVA	Analysis of Variance
APX	Ascorbate peroxidise
BAP	6-benzylaminopurine
BSA	Bovine serum albumin
BTBCB	Balloon type bubble column bioreactor
CAT	Catalase
cc	Centimetres cubed
cv.	Cultivar
dH_2O	Distilled water
DO	Dissolved oxygen
DPPH	1,1-diphenyl-2-picrylhydrazyl
EDTA	Ethylenediaminetetra acetic acid disodium salt
g	Gram
g	Gravity force
$g\ l^{-1}$	Gram perlitre
GPx	Glutathione peroxidise
H_2O_2	Hydrogen peroxide
HCl	Hydrochloric acid
IAA	Indoleacetic acid
MDA	Malonaldehyde
mg	Milligram
ml	Mililitre
MS	Murashige & Skoog
MT	Million tonne
NAA	1-naphthaleneacetic acid
NaOH	Sodium hydroxide
NBT	Nitro blue tetrazolium
O_2	Oxygen
O^{2-}	Superoxide anion

OH^-	Hydroxyl ion
OUR	Oxygen Uptake Rate
PMSF	Phenylmethylsulfonylfluoride
POD	Peroxidase
PrxR	Peroxiredoxin oxidoreductase
PVPP	Polyvinylpyrrolidone
ROS	Reactive oxygen species
SCV	Settled cell volume
SOD	Superoxide dismutase
TCA	Trichloroacetic acid
TMEDA	Tetramethylethylenediamine
UV	Ultraviolet
v/v	Volume / volume
w/v	Weight / volume
Zeatin	4-hydroxy-3-methyl-trans-2-butenylaminopurine
µl	Microlitre
µM	Micromolar
µm	Micrometer
μ_{max}	Specific growth rate

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1.0 Introduction

Banana (*Musa acuminata*) is one of the important staple fruit crops in the tropical region and developing countries as its nutritional status is higher than other common tropical fruits (Sultan *et al*., 2011). In Malaysia, *M. acuminata* cultivar 'Berangan' (AAA) is commercially cultivated due to its flavour and good storage qualities. Traditionally, banana is propagated using field-collected suckers or corm. However, this method is slow as only 5 to 10 suckers are produced per year (Makara *et al*., 2010) due to its long natural life cycle, sterility and polyploidy characteristics. Moreover, it is easy to transmit numerous diseases from old to new banana plantations along the generation route (Msogoya *et al.*, 2011). Thus, there is a need to establish an efficient and less labour intensive method for large-scale production of good quality 'Berangan' in order to satisfy the increasing demands.

Among the tissue culture techniques, somatic embryogenesis has been adapted as an alternative method to mass produce disease-free planting materials. It has a potential to allow cryopreservation and to provide a valuable tool for genetic improvement programmes (Ma *et al*., 2012).A low percentage of somaclonal variation is also an additional advantage of this technique (Borém and Fritsche-Neto, 2014). Furthermore, this method has the advantages of scaled-up propagation using bioreactor (Shibli *et al.*, 2012). Over the years, somatic embryogenesis has been reported for more than 500 plant species, including oil palm, citrus, corn and coffee (Bhojwani and Dantu, 2013). Nevertheless, only a few banana cultivars have been cultured via embryogenesis, such as 'Bluggoe' (*Musa* ABB) (Dhed'a *et al*., 1991), 'Grande Naine' (*Musa* AAA) (Becker *et al*., 2000), 'Rastali' (*Musa* AAB) (Ganapathi *et al*., 2001) and 'Mas' (*Musa* AAB) (Jalil *et al*., 2003) using different types of explants. Among the explants tested,

immature male inflorescences remain the most responsive explant to produce embryogenic callus (Youssef *et al*., 2010).

Traditionally, mass propagation of embryogenic cell suspension cultures is carried out in shake flasks. However, this method requires a lot of space and high electricity consumption. Besides, the parameters to support growth of cells are limited by media composition, temperature and light conditions. These limitations can be overcome by using bioreactors where other parameters can be controlled. Optimum plant growth conditions can be easily achieved in a bioreactor by regulating various chemical and physical factors, including mixing, gaseous composition, efficient oxygen transfer, pH and hydrodynamic forces in bioreactors (Dong *et al.*, 2013). To date, only a few studies have been reported on micropropagation of banana cell cultures using bioreactor. For instance, Kosky *et al.*(2002) propagated tetraploid banana hybrid (FHIA-18) using 2 l CMF-100 (CHEMAP AG) bioreactor and the plants produced were reported to be phenotypically normal.

The critical cell culture conditions, which impact cell growth, plantlet productivity and quality, were optimised and controlled during bioreactor operation. Previous studies indicated that sucrose concentration, inoculum size and medium pH greatly affected cell growth performance (Ferri *et al.*, 2011). Therefore, the present study was carried out to evaluate cell growth and regeneration ability of banana cell suspension cultivated in laboratory scale conventional shake flasks and balloon type bubble column bioreactor (BTBCB).

The objectives of this study were:

1. To determine the regeneration ability of male flower clusters for the initiation of embryogenic cell suspension
2. To optimise the culture parameters of banana embryogenic cell suspension culture grown in shake flasks
3. To investigate the effect of pH-controlled Balloon Type Bubble Column Bioreactor (BTBCB) on the growth of cell suspension cultures
4. To investigate the activities of stress induced enzymes and regeneration ability of banana suspension cultures

2.0 Literature review

2.1 Origin and distribution of banana

Bananas (*Musa* spp.) are perennial and monocotyledonous herbs that belong to the family of *Musaceae* and Zingiberales order (Robinson and Saúco, 2010). There are two genera in the Musaceae family i.e. *Musa* and *Ensete*. According to Consultative Group on International Agricultural Research in 2000, it was believed that bananas originated from Asia and the genus *Musa* are thought to be the first to be domesticated. It was also suggested that distribution of banana plants from Asia to other parts of the world was by transport of vegetative planting material by man, and somatic mutations gave rise to further diversification (Robinson and Saúco, 2010).

According to Stover and Simmonds (1987), the genus *Musa* comprises of over 50 species of edible bananas and plantains. Of these, *Musa acuminata* (AA) and *Musa balbisiana* (BB) are the two main species of *Musa*. Most edible bananas in the market are triploids and hybrids of *M. acuminata* (AA) and *M. balbisiana* (BB). Currently, there are around 20 cultivars, including 'Dwarf', 'Cavendish', 'Robusta', and 'Grande Naine', cultivated at a commercial scale. Of these, 'Cavendish' sub group (AAA) is the most popular species in the international trade due to its nutritional value (Robinson and Saúco, 2010).

Bananas are one of the important fruit sources in tropical region (Nakasone and Paull, 1999). In several African countries, people consume bananas as daily diet due to its dietary value and availability. In 2012, the global gross banana export increased 7.3 % to a new high at 16.5 million tonnes compared to 2011 (FAOSTAT, 2014).India is the world largest producer for banana with an annual production of 31.9 million tonnes (MT) in 2010, followed by China and the Philippines (FAOSTAT, 2011). Banana has

now been classified as the top ten commodities and second fruit crop in the world in terms of production (FAOSTAT, 2011).

2.1.1 Bananas in Malaysia

Banana is a non-seasonal fruit available throughout the year. Normally, bananas are eaten fresh, cooked or processed into preserves food. In Malaysia, bananas contribute to 10 % of the total planted area in Malaysia (Roff *et al*., 2012). Johor is prominent (7,161 ha), followed by Pahang (3,927 ha) and Sarawak (3,929 ha) (Husain and William, 2011). Malaysian government listed banana as one of the fifteen fruit types in the Third National Agricultural Policy (1998-2010) prioritised for commercial cultivation (Ministry of Agriculture, 1999) and also one of the six fruits for development under National Key Economic Area for Entry Point Project. To meet the increasing demand, good agricultural practices have been implemented to improve the banana yield. From 2005 to 2009, the production of banana in Malaysia increased tremendously from 250,000 to 280,000 MT.

In Malaysia, there are about 50 banana varieties, including ‘Berangan’, ‘Mas’, ‘Rastali’, ‘Raja’, ‘Awak’, ‘Abu’, ‘Nangka’, and ‘Tanduk’ (Jalil *et al*., 2003). Of these, ‘Cavendish’ (AAA) and ‘Berangan’ (AAA) are widely planted due to its flavour and good storage qualities (Chai *et al*., 2004) and contribute to about 50 % of the total production. Local farmers prefer ‘Berangan’ compared to ‘Cavendish’ due to its popularity, market value, high bunch weight and availability of planting materials.

2.1.2 Problems of banana cultivation

Although banana industry has undergone spectacular and successful development in the past few years, the yield of bananas affected by biotic and abiotic stresses continues to be a significant challenge. Similar to other plant species, bananas are easily affected by climate change and natural disasters, such as floods, typhoon and droughts. For instance, around 1,500 acres of banana in two districts of Bihar, India, were destroyed by floodwaters in September 2013 (IANS, 2013). In another incident, about 75 % of the banana in Queensland was destroyed by tropical cyclone Yasi in 2011 and causing damage to banana crop worth $350 million (Tadros, 2011).

Bananas are also prone to pest and disease attacks, such as fungi, virus, insects and nematodes. Pests and diseases have caused serious damage to banana plantation worldwide. For instance, banana bunchy top virus which was first reported in 1889 in Fiji has been reported to affect the banana growth (Robinson and Saúco, 2010). This disease dampened the banana production during 1990s as the affected plants will become stunted and have bunchy leaves at the top. No fruits will be produced on the infected plants, and the new suckers produced will also be infected.

Another disease known as Panama disease or *Fusarium* wilt which is caused by *Fusarium oxysporum* f. sp. *cubense* poses a serious threat to the banana plantation. *Fusarium* wilt was first reported in Australia in 1874 (Pegg and Langdon, 1987). This fungus disrupts the translocation and systemic foliage of plants which later caused the collapse of the pseudo-stem. The *Fusarium oxysporum* race 1 pathogen wiped out the dominant cultivar, 'Gros Michel', in the 1950s, and caused enormous economic loss. Around 100,000 acres of banana was destroyed due to this disease in the American continent (Jeger *et al*., 1995). Plantation affected by Panama disease will be inaccessible

for any planting of banana as the fungus will stay dormant in the soil for more than 30 years. Since then, new strain of disease resistant cultivars, 'Cavendish', has been introduced.

In Malaysia, bananas are mostly affected by Panama, Moko and black Sigatoka diseases. The 'Cavendish' plantation in Malaysia was found to be infected by another version of the soil borne fungus, *Fusarium oxysporum*, which was named Tropical Race 4 (Chai *et al*., 2004). Since this pathogen can be spread by soil and water and is resistant to chemical and fungicide, diseases as such will remain to be solved and thus making the demand for clean planting materials to significantly increase.

Bacterial diseases in banana can be divided into two general types, vascular infection caused by *Pseudomonas solanacearum* and soft rotting caused by *Erwinia* species (Jeger *et al*., 1995). In Malaysia, Moko disease or bacterial wilt was first observed in Pontian, Johor, in 1997 after a serious flood. This disease is caused by *Ralstonia solanacearum*. *R. solanacearum* poses a serious problem as it can be spread by water, soil and insects and remain active in the soil for two years. The leaves of infected banana will turn yellow, wilted and finally necrotic. A recent survey carried out by Department of Agriculture (www.portal.doa.gov.my/perpeta/images/banana.pdf), covering 3,212 out of total area of 8,000 ha plantation in Johor, showed that 60.7 % and 23.5 % of banana was infested by Moko and Panama diseases. Examples of major banana diseases in Malaysia are listed in Table 2.1.

Table 2.1: Major diseases of banana in Malaysia (Tengku Maamun, 2011)

Disease name	Causal agent	Type of disease
Moko disease	*Ralstonia solanacearum* race 2	Bacteria
Panama disease	*Fusarium oxysporum* f. sp. Cubenese	Fungus
Yellow Sigatoka	*Mycosphaerella musicola*	Fungus
Black Sigatoka	*Mycosphaerella fijiensis*	Fungus
Cordana Leaf Spot	*Cordana musae*	Fungus

2.2 Tissue culture of banana

Since banana is a highly sterile (seedlessness) and polyploidy plant (Stover and Simmonds, 1987), therefore, genetic improvement through conventional breeding is difficult (Tenkouano and Swennen, 2004). The commercial banana available in the market is mostly triploid and this makes the plant sterile as problem arises during meiosis due to uneven numbers of chromosomes. Most cultivated bananas are parthenocarpic i.e. they are normally propagated vegetatively (Heslop-Harrison and Schwarzacher, 2007). This means that a sucker or shoot will only grow when the mother plant is going to dead. Low germination rate has also led to the low production of planting materials. Thus, biotechnology approaches such as tissue culture and genetic engineering are important part in improving banana yield.

Plant tissue culture offers numerous advantages compared to conventional method as it could mass produce disease-free varieties in a short period. One million plants can be generated from a single explant per year. This technique provides mass production for rare species and also plants with low multiplication rate. In addition, micropropagation requires very small amount of starting material and this may not always be possible *via* conventional breeding. In some seasonal countries, plants can only be planted during some of the time in the whole year. With plant tissue culture,

plants can be planted independent of the seasons. This technique is useful for the propagation of bananas and plantains (Vuylsteke *et al.*, 1997).

In the past, Cox *et al*. (1960) successfully cultured zygotic embryos and Ma and Shii (1974) were the first to report banana plantlets production using *in vitro* shoot tip. However, there are some limitations for this technique, including vitrification, somaclonal variation and production of abnormal plantlets.

Somaclonal variation may arise during tissue culture where not true-to-type plants are produced. 3 – 5 % of somaclonal variation in any type of micropropagation programme is considered acceptable (Sahijram *et al*. 2003). However, in the case of banana tissue culture, somaclonal variation from 0 to 69.1 % had been observed in various types of bananas and plantains and 6 to 38 % for 'Cavendish' cultivars (Hwang and Tang, 2000). In general, *in vitro*-derived banana plantlets are usually exhibit 6 % of somaclonal variation (Martinez *et al.*, 1998). This might be the types of starting explants used, genotype fidelity, and *in vitro* culture conditions (Sahijram *et al*. 2003). Although some variants may be inferior to the mother plant, but many are of undesired traits.

Tissue culture is also useful in producing clonal bananas for breeding purposes (Al-Amin *et al.*, 2009). Propagation of economically important crops *via* direct regeneration without intermediate callus phase is desirable as it can produce high number of true-to-type plantlets (Resmi and Nair, 2007). In contrast, high frequency of variants is usually produced through indirect regeneration pathway which involves intermediate callus stage (Martin *et al*., 2006; Ray *et al*., 2006).

Different types of explants have been used to establish *in vitro* banana culture. Of these, shoot tip is the most commonly used explant (Kulkarni *et al.*, 2006). For example, the apical meristem of banana suckers can be used to induce direct regeneration, whereas male inflorescence can be used to induce direct and indirect regeneration. It is easier to produce clean culture from inflorescence since the banana suckers are highly contaminated with soil microorganisms (Resmi and Nair, 2007; Darvari *et al*., 2010). Furthermore, no somaclonal variation was found when male floral meristems were used (Harirah and Khalid, 2006).

2.2.1 Somatic embryogenesis

Somatic embryogenesis is the process whereby a single cell or a group of cells regenerate to non-zygotic embryos which later become a complete plant (Radzan, 1993). This process needs only a little amount of cells to produce a large amount of clonal cells in a short time (Cote *et al*., 2000). The first somatic embryogenesis was reported on carrot (*Daucus carota*) by Steward (1958). In banana, explants such as meristem and inflorescences were used to produce somatic embryos (Divakaran and Nair, 2011; Kulkarni and Bapat, 2013; Remakanthan *et al*., 2013).

Many factors have been reported to affect the efficiency of embryogenesis, such as type of explants and growth regulator used as well as genotype (Slater *et al*., 2003). Explants containing meristemic cells, such as inflorescence, young root, hypocotyls segments and petioles, usually produced more somatic embryos compared to mature tissues. Vasil (1987) found that low somatic embryo was formed from the mature and differentiated tissue, whereas immature cells and zygotic embryos generated high number of somatic embryos in monocot plants. Similar finding has been reported by Raj

and Vasil (1995), where embryogenesis can only derived from meristemic or immature cells in monocotyledon plants.

Genotype is another factor affecting somatic embryogenesis. For example, *Medicago truncatula* cv. 'Jemalong' has been shown to form somatic embryos 500 times greater compared to other genotypes (Nolan *et al*., 2003). Besides genotype, culturing explants in a suitable medium with optimal pH is also important to generate somatic embryo. Bhojwani and Dantu (2013) reported that 70 % of the cultured explants successfully produced somatic embryos in Murashige and Skoog (MS; 1962) basal medium and modified MS medium.

Plant growth regulators are also essential in initiating somatic embryos. Auxin is an important growth regulator involved in plant growth and development (Cooke *et al*., 1993). Importance of auxin in inducing somatic embryogenesis in carrot was demonstrated by Halperin and Wetherell (1964). It induces cell division, cyto-differentiation and embryogenic differentiation (Bhojwani and Dantu, 2013). Among the auxins, synthetic auxin such as 2,4-dichlorophenoxyacetic acid (2,4-D) has been commonly used to induce callus in many plant species (Bhojwani and Dantu, 2013). Combination of auxin and cytokinin at a suitable concentration could help to generate somatic embryo.

Somatic embryogenesis in *Musaceae* was first reported by Cronauer and Krikorian (1983). The authors induced somatic embryos from cell suspension cultures derived from apices. Since then, many studies have demonstrated the possibility of inducing somatic embryogenesis using female flowers (Grapin *et al*., 2000), immature

male inflorescence (Cote *et al.*, 1996; Khalil *et al*., 2002), and zygotic embryos and male flower bud primordial (Navarro *et al*., 1997).

Cell suspension culture derived from embryogenic callus was successfully established using immature male inflorescences of *M. acuminata* cv. 'Mas' (Jalil *et al.*, 2003). In recent years, a modified method for establishing cell suspension culture in 'Grande Naine' (AAA) using immature male inflorescence has been reported by (Pérez *et al*., 2008). The authors inoculated the male inflorescence in liquid culture medium to produce somatic embryos.

Banana is a recalcitrant plant that has little response in forming somatic embryos compared to other plant species, such as cassava and the ornamental bleeding heart (Strosse *et al*., 2006). Besides, the incubation period for explants to form somatic embryos is long and is varied in different cultivars (Table 2.2).

Table 2.2: Duration for somatic embryo formation in different banana cultivars from male inflorescences

Cultivar type	Duration needed for somatic embryo formation (months)	References
Musa acuminata "Lal Kela" (AAA)	2	Meenakshi *et al*. (2011)
Musa paradisiacal Linn cv. 'Da Jiao' (ABB)	5 - 6	Dai *et al*. (2010)
Musa acuminata cv. 'Mas' (AA)	5 - 6	Jalil *et al*. (2003)
Musa spp. cv. 'Dwarf Brazilian' (AAB)	2	Khalil *et al*. (2002)
Musa sp. 'FHIA-21' (AAAB)	3 - 4	Daniels *et al*. (2002)
Musa spp. cv. 'Grande Naine' (AAA)	5 - 6	Assani *et al*. (2001)

2.2.2 Cell suspension culture

Cell suspension culture is derived from undifferentiatied embryogenic friable calli after transferred into liquid medium (Bhojwani and Dantu, 2013). Continuously shaking of the medium allows the separation of calli clumps into smaller cell aggregates or single cells as well as nutrient and gaseous exchange.

Cell suspension culture has been widely used in plant tissue culture since it can produce higher cell growth rate than callus culture (Walton *et al.*, 1999). Established embryogenic cell suspension could also serve as a tool for genetic transformation, mutation breeding and protoplasts production (Strosse *et al*., 2005).

The duration to establish banana cell suspension is depended on explants used. Strosse *et al*. (2006) reported that shoot meristem tissue required 14 to 42 months to

produce cell suspension culture, whereas it only took 5 to 14 months using scalp. There are two types of suspension cultures, namely batch- and continuous culture. In batch culture, cells are normally cultured in shake flasks and the transferred to a fresh medium after certain period of cultivation. Batch culture normally shows growth curve with sigmoid pattern. For continuous culture, fresh medium is usually added to the culture from time-to-time. This is usually performed in a bioreactor with automated control for a long period of time.

2.2.3 Factors affecting suspension culture

There are many factors affecting the quality of a suspension culture, such as medium, initial inoculum density and light intensity. Therefore, it is essential to optimise the culture conditions. Many studies indicated that cell growth is greatly affected by the medium, nutrients and plant growth regulators used. For example, Baque *et al*. (2013) found that higher number of adventitious roots of *Morinda citrifolia* was generated when cultured on 0.5× Murashige and Skoog (MS) basal salts compared to 1× and 1.5× MS medium. In addition, high concentration of MS salt was found to cause oxidative stress and inhibit the root growth. Hahn *et al*. (2003) reported that full strength MS medium was suitable to promote root growth in *Panax ginseng*, whereas ¾ strength MS was the best medium to promote the production of ginsenoside.

Carbon sources, such as sucrose, glucose and fructose, have also been reported to affect the growth of cell suspension (Rao and Ravishankar, 2002). Although sucrose is widely used as carbon source, however, some studies indicated that fructose or glucose has greater effect on the cell growth. For instance, production of catharantine compounds in *Catharanthus roseus* culture was increased when sucrose was replaced by fructose (Kim *et al*., 2001). Cui *et al*. (2010) reported that 3 % sucrose was suitable

to increase the biomass of *Hypericum perforatum* L. However, sucrose concentration higher than 5 % was found to inhibit the production of biomass due to high external osmotic pressure.

Initial inoculum density has been known to affect the interactions of cell-to-cell and cell-to-medium and cell growth (Aly *et al*., 2010). Hence, it is important to investigate the optimum initial inoculum density in order to maintain the cell-to-cell communication but not to create competition among the cells. Low inoculum densities (5 and 10 g l^{-1}) have been reported to inhibit root growth of *Morinda citrifolia* (Baque *et al*., 2013). Similar results have been reported in *Panax ginseng* and *Catharanthus roseus* cell cultures, in which high inoculum density was found to increase the production of saponin and ajmalicine.

Besides initial inoculums, light intensity also affects the cell growth and development (Heo *et al*., 2002). Under light condition, the growth rate of *Hyoscyamus muticus* cell suspension culture was higher compared to dark condition (Aly *et al*., 2010). Light intensity has also been found to affect the biomass of *Morinda citrifolia* (Baque *et al*., 2010).

2.3 Bioreactor

Since the discovery of plant tissue culture, many types of plants including ornamental flowers, vegetables and fruits have been cultured vegetatively worldwide. Approximately 600 millions of plants were propagated per annum (Altman and Loberant, 2000). Currently, *in vitro* tissue culture is commercially propagated using agar or liquid medium in case of cell suspension. This method, however, is time and space consuming and labour-intensive. Utilisation of shake flasks to mass produce

plantlets usually requires a lot of space and high electricity consumption. To meet these challenges, an alternative technology for the large-scale production of economically important crops is required.

Bioreactor has been considered as a promising tool as it allows mass propagation of plant cultures under controlled environment. It is defined as "a self-contained, sterile environments which capitalises on liquid nutrient inflow and outflow systems, designed for intensive culture with maximal opportunity for monitoring and control over micro environmental conditions (agitation, aeration, temperature, dissolved oxygen, and pH)" (Paek *et al*., 2005). This system is simple, cost saving and could be used to produce biomass, plantlets, metabolites, and enzymes in a short time (Paek *et al*., 2005; Anne Kathrine Hvoslef-Eide, 2005). In addition, setting up a bioreactor and its operation time are lesser compared to conventional culture system. For instance, inoculation of a bioreactor needs only 45 minutes, whereas agar culture needs 1250 minutes for the same amount of culture (Table 2.3).

Recent advancement of bioreactors has enabled researchers to monitor the growth parameters, such as pH, aeration and temperature, effectively (Takayama and Akita, 1994; Lee and Paek, 2012) and could be scaled-up to 2000 litres (Paek *et al*., 2005). At present, there are several types of bioreactors, namely stirred tank reactor, bubble type reactor, and column type reactor, used in plant tissue culture (Sajc, 2000; Paek *et al*., 2005; Gupta and Ibaraki, 2006; Chakrabarty *et al*., 2007).

Table 2.3: Comparison of *Spathiphyllum* culture in bioreactor and agar culture (Takayama and Akita, 2006)

Items		**Bioreactor**	**Agar culture**
Equipment			
	Vessel volume	20 l	500 ml
	Medium volume (l/vessel)	16.6 l (liquid)	100 ml (agar)
	Number of vessels	6	1000
Numbers of inocula used for subculture		96 test tubes	150 test tubes
Culture period		90 days	60 days
Culture space		0.5 m^3	36 m^3
Number of fluorescent lamps (40 W)		6	30
Labour			
	Operation time	200 min	2500 min
Transplanting		1800 min	1800 min

2.3.1 Types of bioreactor

Bioreactors can be divided into mechanically agitated and non-agitated bioreactors (Paek *et al.*, 2005) as shown in Figure 2.1. Stirred tank bioreactor is not suitable for plant cell culture as the rotating stirrer and shear force exerted by the agitation may cause the cells to die. Therefore, non-mechanically agitated bioreactors such as air-lift and column bubble bioreactor are commonly used for cell culture.

Air-lift bubble column bioreactor has a simple construction that gives significantly less shear forces, consumes less energy and high mass production at low input rates (Ziv, 2000). However, this system is prone to excessive foaming due to the small diameter of column preventing bubbles to disperse (Paek *et al.*, 2005). Balloon type bubble bioreactor (BTBB) was designed by Paek *et al.* (2005) in order to reduce

the foaming by introducing a wider part of surface area at the top part of bioreactor. Many plant species and secondary metabolites have been produced through this bioreactor. For instance, pilot scale of 300-1000 l has been constructed to produce the biomass from *Panax ginseng*, orchids and other valuable plants (Paek *et al.*, 2005).

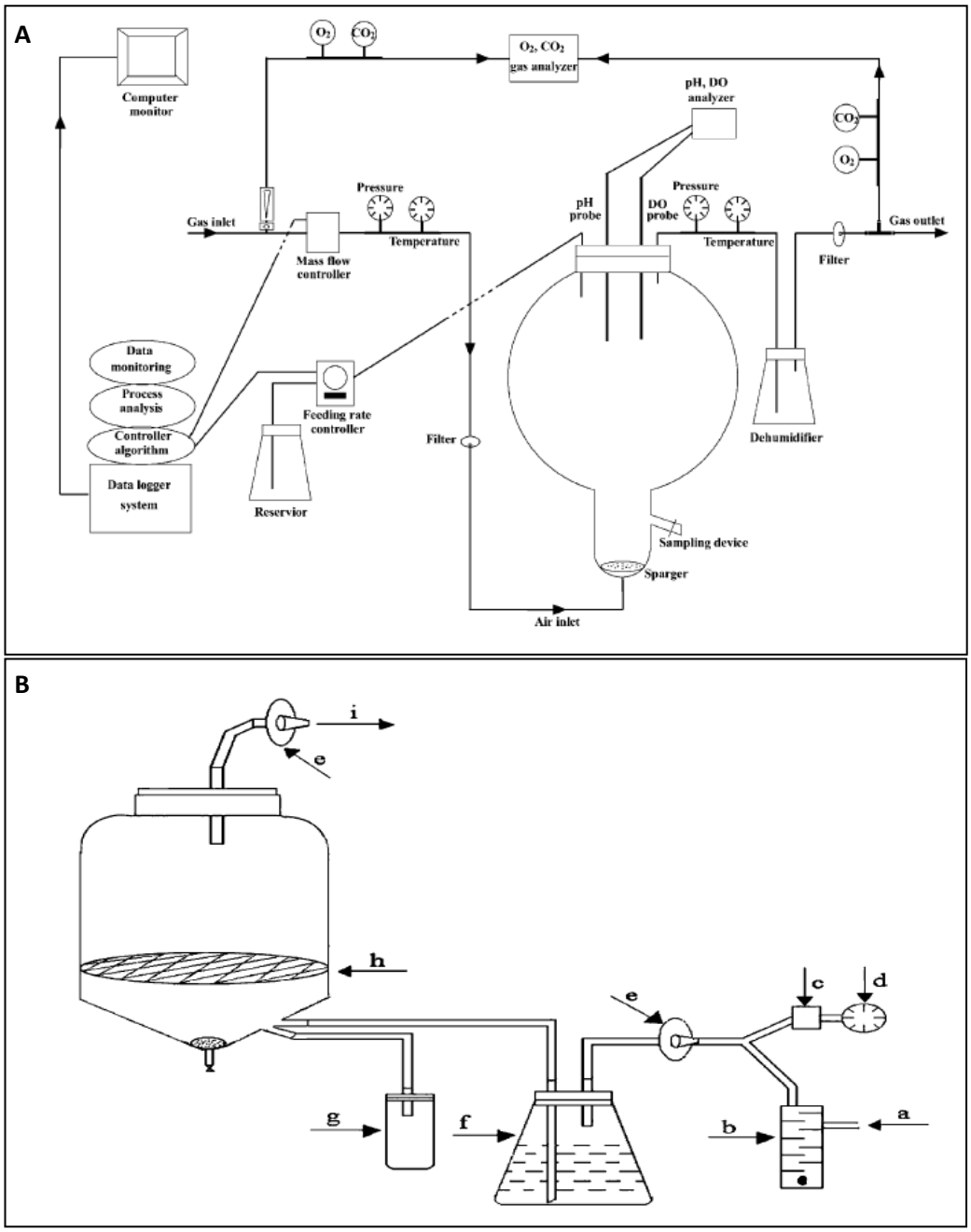

Figure 2.1: Example of bioreactors for plant cell, tissues and organ cultures (Paek *et al*., 2005). (A) Balloon type bubble bioreactor (B) ebb and flood type bioreactor.

2.3.2 Applications of bioreactor

Micropropagation using a bioreactor was first reported in *Begonia* by Takayama and Misawa (1983). In the subsequent years, many studies have been carried out to cultivate plant cell using bioreactors. These include apple, banana, *Begonia,* Boston fern, *Chrysanthemum, Colocasia, Dieffenbachia, Dioscorea, Gladiolus, Hippeastrum, Lilium, Narcissus, Nerine, Ornithogalum, Phalaenopsis, Pinellia, Saintpaulia, Sinningia (Gloxinia), Spathiphyllum* and *Stevia* (Denchev *et al.*, 1992; Takayama and Akita, 1994; Paek *et al*., 2005). Earlier study reported the possibility of producing shoots of *Stevia rebaudiana* in a 500 litres bioreactor (Akita *et al.*, 1994). Dewir *et al*. (2006) also successfully mass propagated *Spathiphyllum cannifolium* using an air-lift bioreactor. In banana, tetraploid banana hybrid (FHIA-18) cells were able to scaled-up to a 2 litres CMF-100 (CHEMAP AG) bioreactor without producing any somaclonal variants (Kosky *et al*., 2002).

Besides producing *in vitro* plantlets, bioreactor has been used to produce pharmaceutically important metabolites in private industries (Sajc, 2000). In nature, secondary metabolites are extracted from whole plants or tissues. However, the yield of these useful metabolites is usually limited by weather, natural disaster, and insufficient planting stocks (Paek *et al*., 2005). This limitation can be overcome by using a bioreactor since the compounds can be continuously produced from the target organs or tissues. For example, roots of *Panax ginseng* were cultured in a pilot scale automated bioreactor of 1000 litres for extraction of ginsenoside (Paek *et al*., 2001). Important secondary metabolites, such as shikonin, ginsenosides and berberine, have also been produced (Bourgaud *et al.*, 2001). Examples of plant cells produced by bioreactor are listed in Table 2.4.

Table 2.4: Bioreactor systems studied for plant cells cultivation

Plant	Type of bioreactor used	Source
Begonia	Air-lift bioreactor	Takayama and Misawa (1983)
Tobacco	Stirred-tank, Air-lift bioreactor	Lee *et al*. (2001)
Banana hybrid (FHIA-18)	CMF-100 bioreactor	Gómez Kosky *et al*. (2002)
Spathiphyllum cannifolium	Air-lift bioreactor	Dewir *et al*. (2006)
***Panax ginseng* (C.A. Meyer)**	BTBB	Wang *et al*. (2012)
***Eleutherococcus senticosus* Maxim**	Bubble column bioreactor	Yang *et al*. (2012)
***Anoectochilus formosanus* (endangered orchid)**	BTBB	Yoon *et al*. (2007)
Apple rootstock M9 EMLA	Ebb and flood bioreactor	Chakrabarty *et al*. (2007)
Chrysanthemum	Air-lift column bioreactor	Kim (2005)
Grapevine	Air-lift column bioreactor	Tapia *et al*. (2009)
Lilium	BTBB	Lian *et al*. (2003)
Potato	BTBB, column-type bioreactor	Piao *et al*. (2003)
Hypericum perforatum L	BTBB	Cui *et al*. (2010)
Eleutherococcus sessiliflorus	BTBB	Shohael *et al*. (2005)
Panax ginseng	BTBB	Bae *et al*. (2006)

Bioreactor has also been used to produce somatic embryos in coffee, alfalfa, carrot, rubber, and sweet potatoes (Etienne *et al*., 2006). Although bioreactor greatly increased the biomass of cell cultures, some authors found that the biomass was decreased after transferred from shake flask to bioreactor. This might be due to the stress and strong agitation applied in the bioreactor. Pan *et al*. (2000) reported a sharp decrease of secondary metabolite, taxuyunnanine C, in *Taxus chinensis* when transferred from shake flask to bioreactor. This attributed to the insufficient oxygen supply, high shear stress and decrease of gaseous metabolites. However, the production of taxuyunnanine C increased tremendously after adding ethylene gas through inlet air, suggesting the importance of optimising culture conditions in the bioreactor system.

2.3.3 Problems of bioreactor culture

Although bioreactor has undergone a spectacular development, various problems such as decrease growth rate and cell productivity, somaclonal variation and limited photoautotrophic growth, continue to be significant limitations (Bourgaud *et al*., 2001). Vitrification often occurs when plant cells are cultured in liquid medium (Vasil, 1994). Furthermore, high humidity in the bioreactor has been found to increase the possibility of forming hyperhydricity plantlets. Constant immersion of shoots in the culture media for a long period might retard the plant growth.

Vitrification or hyperhydricity is also caused by excessive hydration and low lignifications in plants. Thus, several parameters, such as improving the carbon dioxide content and air exchange in the medium, need to be optimised in order to reduce the frequency of hyperhydricity. This problem has been resolved by using temporary immersion system, such as ebb and flow (Akita and Takayama, 1994), Rita™ System (Teisson and Avard, 1999), and twin-flask system (Jimenez *et al*., 1999).

2.3.4 Factors affecting bioreactor culture

There are several factors that must be considered to grow cultures in a bioreactor, including initial inoculum density, oxygen supply, pH, aeration and media components. Controlling of the micro-environment within the bioreactor is essential to maximize the growth of cell cultures or plant biomass.

2.3.4.1 Nutrients

Nutrients availability in a bioreactor is the main factor affecting the growth of cells. Determination of nutrient level is important to maintain cell growth. Besides, oxygen is required for cellular growth and aerobic metabolism of suspended plant cells in *in vitro* culture. Oxygen uptake rate (OUR) can be used as an indicator for monitoring the physiology and oxygen demand of plant cells during suspension culture in a bioreactor. One of the main advantages of bioreactor over shake flask is that transfer of oxygen to the liquid phase is relatively easier through bubbles. Since oxygen is only sparingly soluble in water (0.25 mmol l^{-1}) at 25 ℃, 1 atm, 21 % O_2 in the air, it is necessary to drive the diffusion of oxygen into the aqueous phase to meet the demand of actively growing tissues or cells by modifying the aeration rate, impeller design and speed (Leathers *et al*., 1995).

2.3.4.2 Mixing of media

Mixing of the media and cells within bioreactors is important as oxygen must be transferred to the cells at fastest rate to cut down cell stress. The nutrients and oxygen must be circulating always for the cells to uptake and grow (Leathers *et al*., 1995; Sajc, 2000). Mixing can be done by impeller or bubbles. For plant cell tissue culture, impellers or stirrer are not suitable as they normally exert high shear stress on the cells. Mixing by introducing bubbles from the bottom of bioreactors is preferable but the

mixing rate must be sufficient to the cells and not too high to cause stress on them. Good aeration in the bioreactor can produce good quality cells that are easily acclimatized and thus solve the problem of vitrification of plants.

2.3.4.3 Aeration

Lee *et al*. (2006) discussed that aeration volume is important in bioreactor culture as it indirectly affects the agitation of the explants and dissolved oxygen (DO) in the media. High aeration volume in the bioreactor may cause severe foaming around the bioreactor wall and this will affect the cell growth. Even though anti-foam was introduced in recent years, it is seldom used in plant cultures as in some cases, it resulted in reduction of cell growth and harmful for human health (Wongasmuth and Doran, 1994). Aeration and mixing at the (different) optimum rate is important to enhance the production of biomass or secondary metabolites in plants. For example, *Morinda citrifolia* achieved the highest cell growth at 0.3 vvm but maximal production of secondary metabolites was recorded at 0.1 vvm (Ahmed *et al.*, 2008). On the other hand, Lee *et al.* (2006) reported that a step by step increment of aeration volume from 0.05 to 0.4 vvm was needed for the growth of *Gymnema sylvestre* cell suspension culture. Trung Thanh *et al.* (2006) reported that 0.1 vvm was the best aeration volume for *Panax ginseng* root culture and ginsenocide production.

2.3.4.4 Dissolved oxygen (DO)

DO concentration in the media is also important in maintaining cell growth and viability (Leathers *et al*., 1995; Sajc, 2000). Gao and Lee (1992) studied the effect of oxygen supply on genetically modified tobacco cells and found that an increase in the oxygen supply enhanced specific growth rate, maximum cell concentration,

consumption rate of glucose and fructose and GUS protein production yield in shake flask, stirred-tank and air-lift bioreactors.

Mavituna and Buyukalaca (1996) reported that the oxygen uptake rates of *Capsicum annum* L. cv. 'Ace' (pepper) in an air-lift bioreactor was different throughout the developmental of embryogenesis. The authors indicated that the highest oxygen uptake rate was recorded in embryogenic suspension cultures and the lowest was found during embryo maturation (Mavituna and Buyukalaca, 1996). Shigeta *et al.* (1996) demonstrated that about 80 % DO was required during the early phase of carrot embryo development. For *Cyclamen,* 40 % DO in the liquid culture produced significantly high amount of embryos compared to shake flasks or bioreactors with 5-20 % DO (Hohe *et al*., 1999).

2.3.4.5 pH

pH has been known as one of the parameters that greatly influences the rate of somatic embryogenesis in bioreactors (Yu *et al.*, 2001). In *Daucus*, culture medium with pH 4.3 produced high amounts of non-regenerable embryos, whereas cells cultivated in a medium with pH 5.8 produced fewer embryos but recorded higher percentage of embryo development (Jay *et al*., 1994). Precise recording of fluctuations in parameters like the pH using computer controlled data logging will improve the repeatability of complex biological processes, such as plant cell cultivation in bioreactors.

2.4 Reactive oxygen species (ROS)

Reactive oxygen species (ROS) are chemically reactive radicals produced by plants under severe stress conditions. When under abiotic or biotic stress conditions, such as flood, drought, high salinity and pest attack, ROS namely hydrogen peroxides (H_2O_2), superoxide anion (O_2^-) and hydroxyl ions (OH^-) will be accumulated due to the discrepancy between scavenging and production of ROS (Hideg *et al*., 2013).

In plants, enzymatic and non-enzymatic scavenging mechanisms play an important role in scavenging ROS. Excessive ROS will cause damage to proteins, DNA and lipids, and end up in fatality to cells or even the whole plants (Mittler, 2002; Zulfugarov *et al*., 2011). 'Oxidative stress' is normally used to describe the imbalance of ROS towards the pro-oxidative states in plants (Apel and Hirt, 2004). Examples of ROS antioxidant enzyme are listed in Table 2.5.

Table 2.5: Examples of antioxidant enzyme and their reactions

Enzyme	**Reaction catalysed**
Superoxide dismutase (SOD)	$2O_2^- + 2H^+ \leftrightarrow H_2O_2 + O_2$
Catalase (CAT)	$2\ H_2O_2 \leftrightarrow\ O_2 + 2\ H_2O$
Glutathione peroxidase (GPx)	2GSH + lipid hydroperoxide ↔ glutathione disulfide + lipid + 2 H_2O
Dehydroascorbate reductase	2GSH + DHA ↔ glutathione disulfide + ascorbate
L-ascorbate peroxidise	2 L-ascorbate + H_2O_2 + 2H ↔ L-ascorbate + L-dehydroascorbate + 2 H_2O

ROS are usually produced in peroxisomes, chloroplasts, mitochondria, plasma membrane and apoplast. Normally, each organelle consists of more than one enzyme that can scavenge ROS. For example, cytosol contains ascorbate peroxidise (APX),

glutathione peroxidise (GPx) and peroxiredoxin oxidoreductase (PrxR) to counteract H_2O_2 produced (Suzuki and Mittler, 2006). Superoxide dismutase (SOD) was first reported by Scandalios (1993) in maize which contained six distinct isozymes. Abiotic stress in plants will cause increase in SOD to counteract the oxidative stress. Increase in salinity will also cause the up-regulation of SOD in plants. This condition has been observed in barley (Liang, 1999), tomato (Shalata *et al*., 2001) and mulberry (Harinasut *et al*., 2003).

Catalase (CAT) is the main scavenging agent for H_2O_2 in peroxisome. Sudhakar *et al*. (2001) reported that CAT activity in salt tolerant mulberry cultivar S1 was higher compared to salt susceptible cultivar ATP. H_2O_2 is formed in the peroxisomes as a result of photorespiration. However, H_2O_2 will also be formed as a by-product during the β-oxidation of fatty acids. Under normal condition, the H_2O_2 produced will be utilised for cell metabolism.

SOD is found in various parts of the cells and catalyses two O_2^- radicals into H_2O_2 and oxygen (O_2) which are non-destructive to the cells (Scandalios, 1993). The H_2O_2 produced will be scavenged by other antioxidant enzymes, such as CAT and peroxidase (POD). CAT is said to be capable of scavenging large quantity of H_2O_2. H_2O_2 produced away from the peroxisome, e.g. chloroplast, will be scavenged by APX (Asada, 1992). Under normal condition, the scavenging of ROS will be regulated well by these mechanisms during cell metabolism but when the plants are under environmental stresses, ROS will attack vital cell components and affect cell metabolism, which in the end caused cell death (Sakihama *et al*., 2002).

3.0 Materials and methods

3.1 Banana tissue culture

3.1.1 Explant source

Inflorescence male buds of *Musa acuminata* cv. 'Berangan' (AAA) collected from the local market at Seksyen 17, Petaling Jaya, Malaysia, were used as explants to initiate callus (Figure 3.1).

3.1.2 Surface sterilisation and callus induction

The male inflorescences were surface sterilised and cultured according to the procedures of Escalant *et al.* (1994). Immature male inflorescence collected 1 to 10 weeks after flowering was cut into smaller sizes (5-6 cm) by removing its outer bracts (Figure 3.2). Surface sterilisation was carried out by submerging and shaking the explants in 70 % (v/v) ethanol for 15 min inside a laminar airflow cabinet followed by washing three times with distilled water. The male inflorescences were trimmed aseptically to 1-2 cm. A total of 15 immature male inflorescence clusters occupying positions 1 to 15 (first being the flower cluster closest to the meristematic dome) (Figure 3.3) were removed under microscope and inoculated on modified M1 solid medium (Jalil *et al.*, 2003) containing Murashige and Skoog (MS) (1962) basal salts supplemented with Dhed'a vitamins (Dhed'a *et al.*, 1991), 100 mg l^{-1} myo-inositol (Sigma, USA), 1 mg l^{-1} biotin, 1 mg l^{-1} indoleacetic acid (IAA) (Sigma, USA), 1 mg l^{-1} 1-naphthaleneacetic acid (NAA) (Sigma, USA), 4 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) (Sigma, USA), 10 mg l^{-1} ascorbic acid (Sigma, USA), 30 g l^{-1} sucrose (Sigma, USA), and 2 g l^{-1} Gelrite (Duchefa, Netherland). The media were adjusted to pH 5.7 prior to autoclaving at 121 ℃ for 20 min. All cultures were incubated at 25 ± 2 ℃ in dark condition. Morphological changes of initial explants and emergence of callus were observed every two weeks.

Figure 3.1: Male inflorescence of *Musa acuminata* cv. ‘Berangan’.

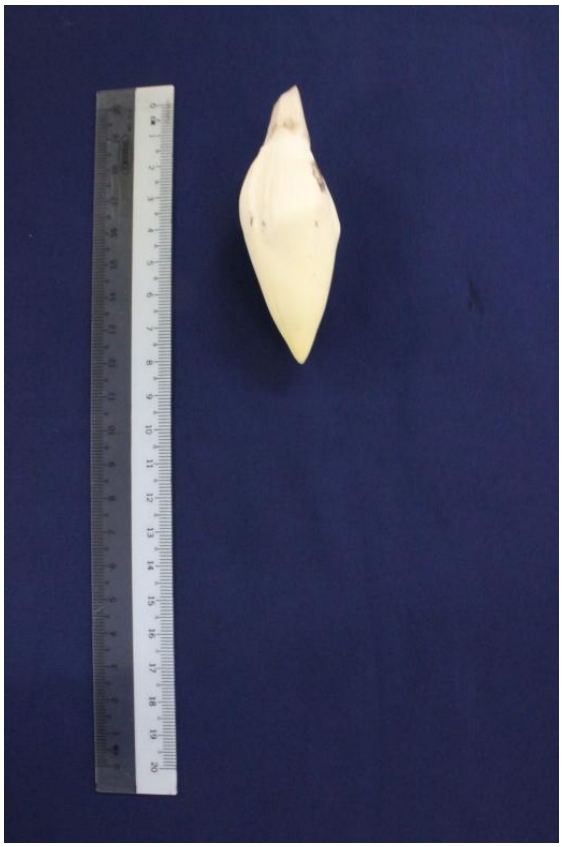

Figure 3.2: Dissected size male inflorescence of *Musa acuminata* cv. ‘Berangan’.

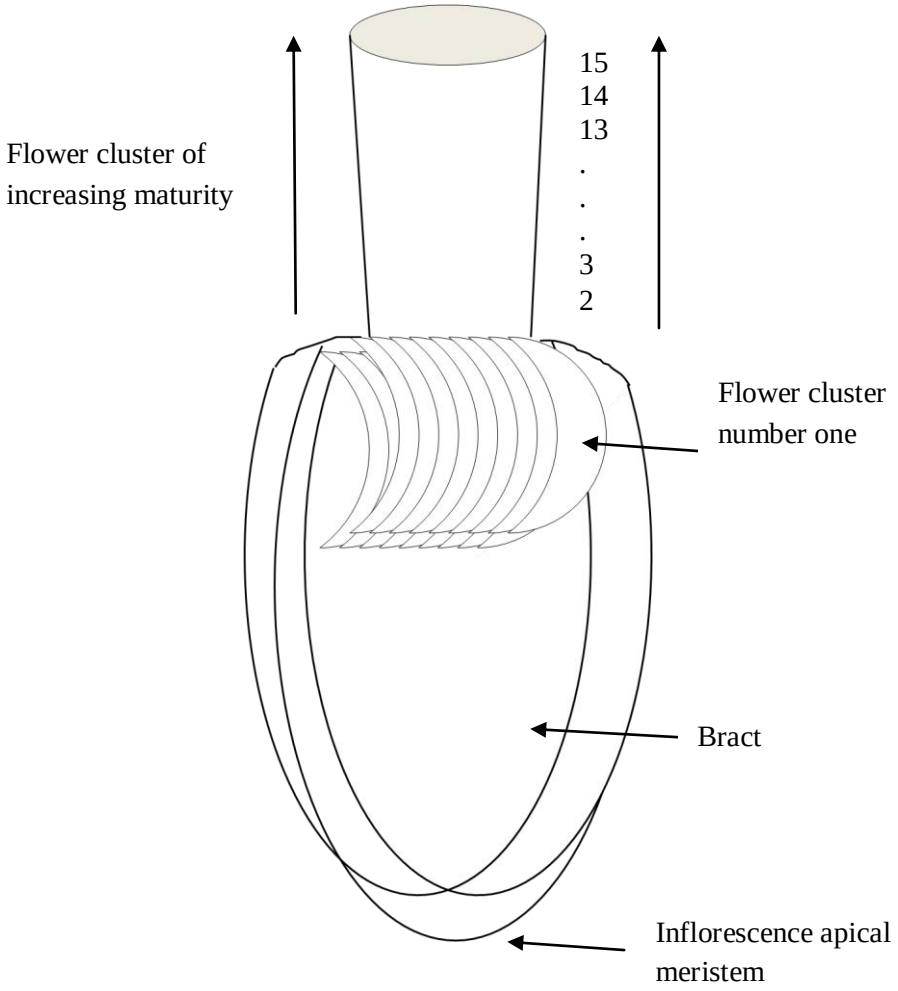

Figure 3.3: Schematic diagram of a male inflorescence.

3.1.3 Establishment and maintenance of cell suspension culture

Cell suspension cultures were established according to Jalil *et al.* (2003). Translucent embryos and yellowish friable embryogenic callus were transferred into 50 ml conical flasks containing 10 ml M2 liquid medium (Côte *et al.*, 1996). This M2 medium consisted of MS basal salts, MS vitamins, 10 mg l^{-1} ascorbic acid (Sigma, USA), 1.1 mg l^{-1} 2,4-D, 250 mg l^{-1} trans-zeatin (Duchefa, Netherland), 30 g l^{-1} sucrose and 100 mg l^{-1} myo-inositol. Zeatin was filter-sterilised using 0.2 µm Millipore filter (Millex-GS, Millipore) and added to the media after autoclaving. The cell suspension cultures were sub-cultured every 10 days by replacing half of the media with fresh media. After 30 days, the suspension cultures were transferred into a 250 ml shake flask containing 50 ml M2 medium. The cell suspension cultures were sub-cultured by replacing 25 ml used medium with an equal volume of fresh medium every 14 days. The cells were sieved by using a metal mesh with 425 µm pore size (Endecotts Limited, England) after two months of culture to eliminate big clumps of cells. The filtrate was sub-cultured every fourteen days. Cell suspension cultures were incubated at 25 ± 2 °C under continuous shaking condition of 90 rpm with a light intensity of 31.4 µmol m^{-2} s^{-1}.

3.2 Media optimisation in shake flask for cell suspension cultures

3.2.1 Effects of sucrose concentration

Established cell suspension cultures were initially inoculated in M2 liquid medium supplemented with different concentrations of sucrose (10, 20 and 30 g l^{-1}) to determine the optimum range of sucrose concentration. The cell suspension was later cultured in extended range of sucrose concentrations (10, 15, 20, 25, 30 and 35 g l^{-1}) to obtain the optimum concentration for cell growth. The growth of cell suspension culture

was measured by settled cell volume (SCV) after 14 days. Specific growth rate and growth yield were calculated using the following formulas:

$$\textit{Specific growth rate, } \mu_{max} \text{ (day}^{-1}\text{)} = \frac{ln\ (SCV\ day\ 14) - ln\ (SCV\ day\ 1)}{14} \qquad (I)$$

$$\textit{Growth yield} = \frac{SCV\ \ day\ 14 - SCV\ day\ 1}{SCV\ day\ 1} \qquad (II)$$

ln= logarithm to the base *e*, where e is an irrational and transcendental constant approximately equal to 2.72.

3.2.2 Effects of initial inoculum density

To determine effects of inoculum density on cell growth, established cell suspension cultures were inoculated in M2 liquid medium supplemented with different initial inoculum densities (1.0, 1.5 and 2.0 ml) in 50 ml of M2 medium, with optimum concentration determined in Section 3.2.1.

3.2.3 Effects of vessel size

Three different sizes of shake flasks were used to determine its effect on cell growth over 14 days. An initial inoculum density of 1.0, 2.0 and 4.0 ml SCV was used in 50 ml (250 ml shake flask), 100 ml (500 ml shake flask) and 200 ml (1000 ml shake flask) M2 medium, respectively. The cell suspension cultures were cultured under optimum sucrose concentration and initial inoculum density determined in section Sections 3.2.1 and 3.2.2. Specific growth rate was determined after 14 days of culture.

3.2.4 Effects of photoperiod

Two different photoperiods (16 h light / 8 h dark and 24 h light) with a light intensity of 31.4 μmol m^{-2} s^{-1} were used to determine its effects on cell growth. Cell suspension was cultured under optimised sucrose concentration and initial inoculum density as determined in Sections 3.2.1 and 3.2.2. Specific growth rate was determined after 14 days of culture.

3.2.5 Growth curve and pH curve of cell suspension cultures

A growth curve of banana cell suspension culture was plotted by using the optimised cultures determined from Sections 3.2.1, 3.2.2, 3.2.3 and 3.2.4. The growth and pH of cell suspension cultures were measured every two days until the cells reached the death phase. Specific growth rate was determined after 14 days of culture.

3.3 Optimisation of selected cultivation variables in balloon type bubble column bioreactor (BTBCB)

The cell suspension cultures were mass propagated in a 5 litre balloon type bubble column bioreactor (BTBCB) fabricated by Fermetec Resources Sdn. Bhd., Malaysia, to study the growth of banana cells in a scale-up environment. Airflow and pH of the medium were recorded by a controller (Figure 3.4). The pH of medium was recorded every two days throughout 14 days of culture. The airflow was supplied by an air pump with a flow rate at 100 ml min^{-1} to achieve a homogenous mixing stage. Initial inoculum density of 20 ml SCV was cultured in 1 litre M2 medium and incubated at 25 ± 2 ℃ under light intensity of 31.4 μmol m^{-2} s^{-1}. The specific growth rate and growth ratio was calculated after 14 days of culture.

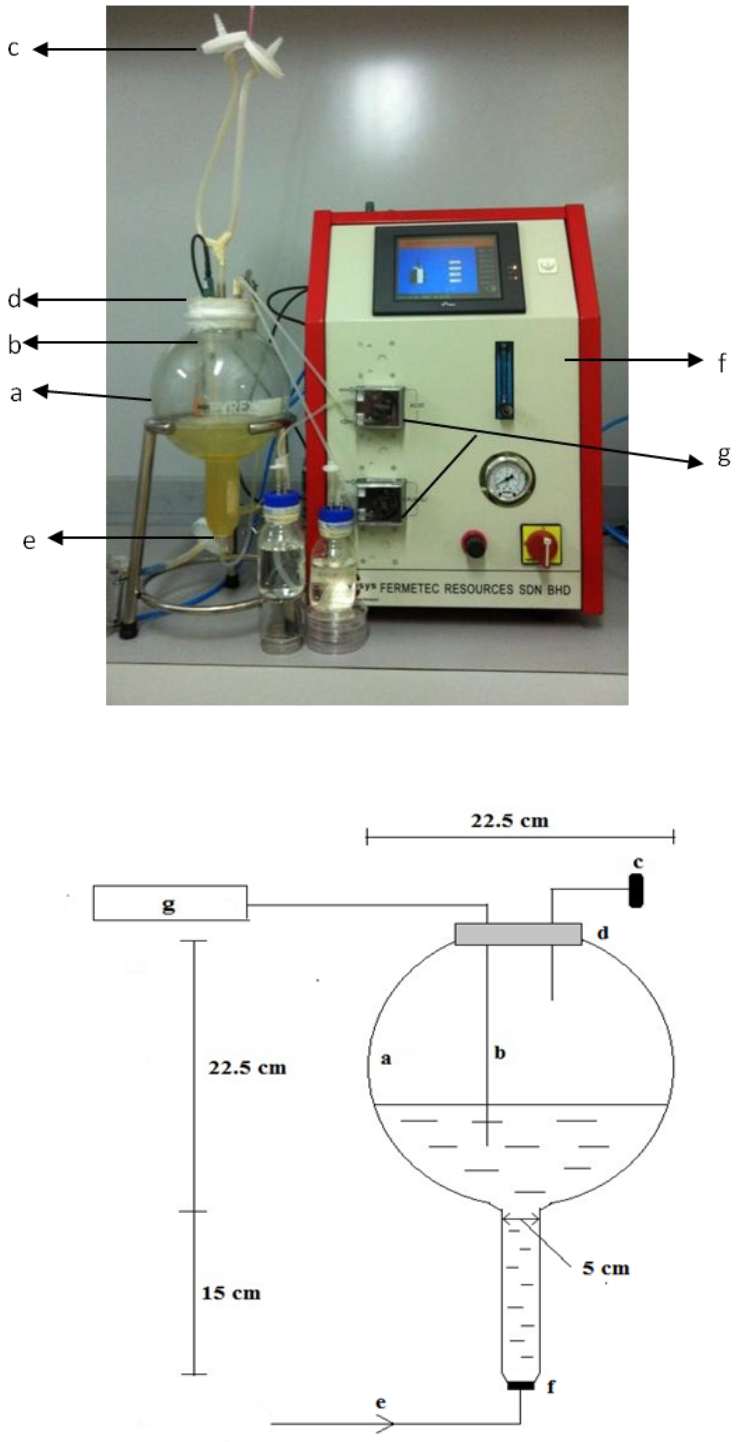

Figure 3.4: Picture (top) and schematic diagram (bottom) of a 5 l balloon type bubble column bioreactor (BTBCB). (a) BTBCB (b) pH meter, (c) filter vent, (d) silicon cap, (e) filtered air inlet, (f) automated controller, and (g) acid and alkali controller.

3.3.1 Effect of controlled pH on cell suspension culture

The medium's pH in BTBCB was regulated at 5.7 with 0.5 M NaOH or 0.5 M HCl delivered using automatic peristaltic pumps controlled by PLC Control System For Fermentation software (Fermetec Resources Sdn. Bhd., Malaysia). The specific growth rate of cells was calculated for each BTBCB every two days over 14 days. The air flow rate was adjusted to 100 ml min^{-1} during cultivation to achieve a homogenous mixing. The cultivation temperature in bioreactors was controlled at 25 ± 2 ℃ under light intensity of 31.4 μmol m^{-2} s^{-1}. The specific growth rate and growth ratio was calculated after 14 days of culture.

3.3.2 Embryo development and regeneration

After 14 days of culture, cell suspension cultures were transferred to solid development medium (M3) containing MS basal salts, MS vitamins, 30 g l^{-1} sucrose and 2 g l^{-1} Gelrite. The cells were sieved through 425 μm mesh size sieve and cell density was adjusted to 20 % SCV in a Falcon tube before culture. 200 μl of the adjusted cell suspension cultures were transferred and cultured on a 70 mm No.1 Whatman filter paper in M3 medium. All cultures were maintained at 25 ± 2 ℃ under dark condition until forming somatic embryos. Developed embryos were then transferred to modified regeneration medium (M4) (Jalil *et al.* 2003) consisting of MS basal salts, MS vitamins, 30 g l^{-1} sucrose, and 8 mg l^{-1} 6-benzylaminopurine (BAP) and cultured at 25 ± 2 ℃ under light condition.

After one month of culture, the regenerated shoots were transferred to M4B medium for further regeneration. M4B medium consisted of the same component as M4 except 8 mg l^{-1} BAP was decreased to 4 mg l^{-1}. The clumps of mature somatic embryos were arranged in 4 rows with 5 clumps each at a distance of 0.5 cm to facilitate the

calculation of percentage of regenerated somatic embryos (Figure 3.5). Each treatment contained 100 clumps per replicate and was repeated twice. The percentage of shoot regeneration was determined by calculating the number of clumps developed into shoots over total number of somatic embryos. Abnormal regenerated plantlets such as stunted plantlets or crystallised leaves were recorded.

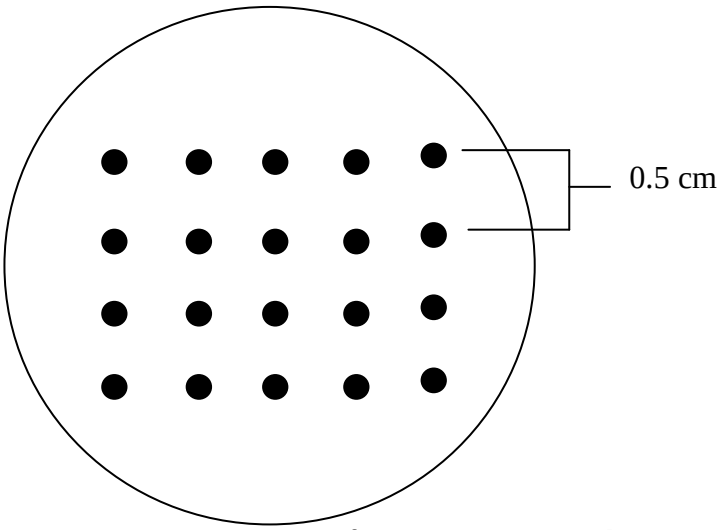

Figure 3.5: Arrangement of mature somatic embryos for regeneration on M4 medium.

3.4 *In vitro* rooting (M5)

Regenerated plantlets were transferred for root induction on M5 medium containing MS basal salts, MS vitamins, 8 g l^{-1} activated charcoal (Duchefa, Netherlands), 30 g l^{-1} sucrose and 2 g l^{-1} Gelrite.

3.5 Determination of protein content and stress induced enzyme activities

3.5.1 Protein extraction

Fresh cells (1 g) frozen in liquid nitrogen were ground into a fine powder in 2.0 ml of 50 mM potassium phosphate buffer (pH 7.0) containing 2 % (w/v) polyvinylpyrrolidone (PVPP) (Sigma, USA) and 1 mM phenylmethylsulfonylfluoride (PMSF) (Sigma, USA) by using pre-chilled mortar and pestle. The mixture was centrifuged at 8,000 × *g* for 5 min at 4 ℃. The supernatant was then transferred to a new tube and centrifuged at the same speed for another 5 min. Protein content was determined by Bradford method (1976) using bovine serum albumin (BSA) as a standard (Table 3.1). All readings were done by using a UV-VIS spectrophotometer (Thermo Scientific GENESYS 10UV-Vis, USA). The BSA standard curve is shown in Figure 3.6.

Table 3.1: Reaction for determining protein content

Chemicals	**Volume (ml)**
Distilled water	0.59
Bradford dye	0.40
Extracted sample	0.01
Incubate for 10 min at room temperature in dark	
Read absorbance at 595 nm	

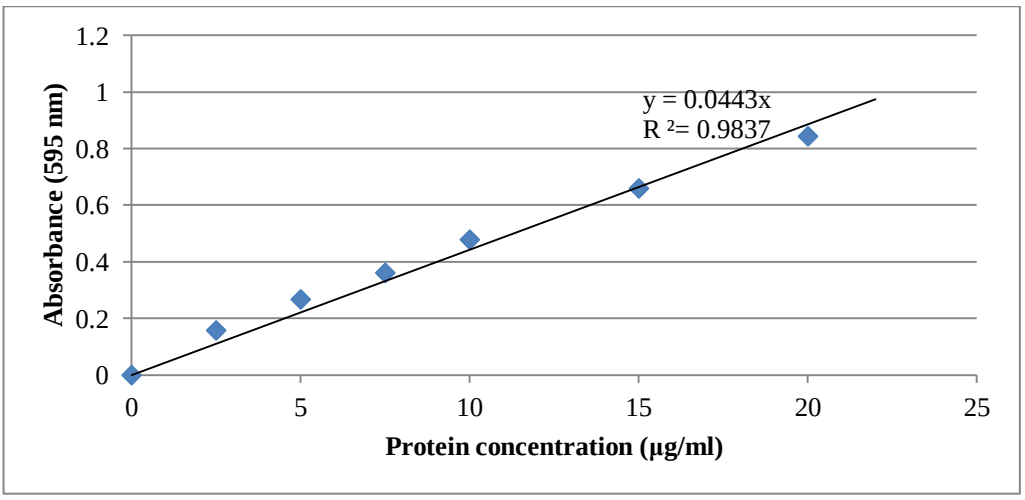

Figure 3.6: Protein standard curve.

3.5.2 Determination of hydrogen peroxide (H_2O_2) concentration

Hydrogen peroxide was measured according to Baque *et al.* (2012). 1.0 g cells fresh weight and 2.0 ml of 0.1 % (w/v) trichloroacetic acid (TCA) (Sigma, USA) were homogenised in liquid nitrogen by using pre-chilled mortar and pestle. The extract was centrifuged at 10,000 × *g* for 15 min at 4 ℃. 0.5 ml supernatant was added into 0.5 ml of 10 mM K-phosphate buffer (pH 7.0) and 1.0 ml of potassium iodide (KI) and incubated for one hour in dark condition. The mixture was measured using a spectrophotometer at 390 nm. A mixture of 1.0 ml of 10 mM K-phosphate buffer and 1.0 ml KI without extracts was used as control. The amount of hydrogen peroxide was determined using H_2O_2 standard curve. Table 3.2 showed the components of reaction mixture for H_2O_2 estimation. H_2O_2 standard curve is as shown in Figure 3.7.

Table 3.2: Determination of hydrogen peroxide (H_2O_2) content

Chemicals	Sample (ml)	Blank (ml)
Extracted sample	0.5	-
10 mM k-phosphate buffer	0.5	1.0
1 M KI	1.0	1.0
Incubate for 1 h in dark		
Read absorbance at 390 nm		

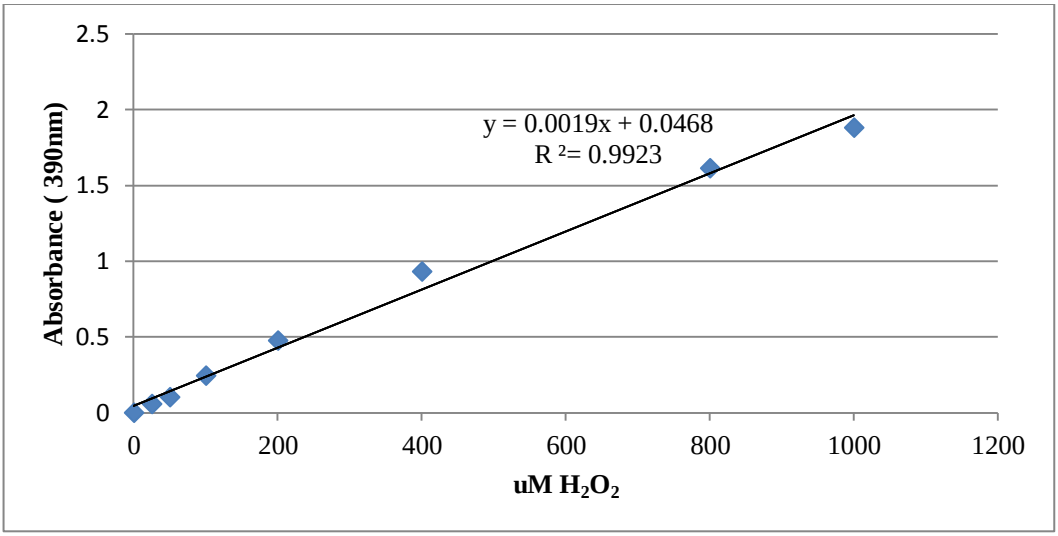

Figure 3.7: Hydrogen peroxide standard curve.

3.5.3 Determination of superoxide dismutase (SOD)

Superoxide dismutase (SOD) activity was measured according to Beauchamp and Fridovich (1971) (Table 3.3). Fresh cells (1 g) frozen in liquid nitrogen were ground into a fine powder in 2.0 ml of 50 mM potassium phosphate buffer (pH 7.0) containing 2 % (w/v) PVPP and 1 mM PMSF. The mixture was centrifuged at 8,000 $\times g$ for 10 min at 4 ℃. The supernatant was transferred to a new tube and centrifuged at the same force for another 10 min. The extracts was added into Na-phosphate buffer (pH 7.8), 10 µm riboflavin, 250 µm nitro blue tetrazolium (NBT) and 1 µm tetramethylethylenediamine (TMEDA) (Sigma, USA) and incubated for 15 min. Ability of SOD to inhibit the reduction of NBT (Sigma, USA) was measured by a

spectrophotometer at 560 nm (25 °C). The unit of SOD (SOD_{50}) is defined as the amount of SOD required to cause 50 % of inhibition of the oxidation. The activity of SOD was calculated by the difference between the sample and blank in one minute over 100 mg of fresh cells i.e. SOD_{50} 100 mg FW^{-1} min^{-1}.

Table 3.3: Determination of superoxide dismutase (SOD) activity

Chemicals	**Sample (ml)**	**Blank (ml)**
Na-phosphate buffer	1.0	1.0
NBT	1.0	1.0
Distilled water	1.94	1.94
Extracted sample	0.05	-
Distilled water	-	0.05
Riboflavin	1.0	1.0
TMEDA	0.01	0.01
Incubate for 15 min at room temperature		
Read absorbance at 560 nm		

3.5.4 Determination of catalase (CAT)

Catalase (CAT) activity was measured according to the modified method of Baque *et al.* (2012) (Table 3.4). Fresh cells (1 g) frozen in liquid nitrogen were ground into a fine powder in 2.0 ml of 50 mM potassium phosphate buffer (pH 7.0) containing 2% (w/v) PVPP and 1 mM PMSF. The mixture was centrifuged at 8,000 × *g* for 10 min at 4 ℃. The supernatant was then transferred to a new tube and centrifuged at the same force for another 10 min. Subsequently, the supernatant was incubated with CAT reaction mixture [100 mM H_2O_2 in 100 mM potassium phosphate buffer (pH 7.0)] for 10 min and then measured at 240 nm (25 °C). 1.0 ml of 2 N sulphuric acid was used to replace extracts in the reaction mixture as a blank. CAT activity was calculated based

on the amount of H_2O_2 being split in one minute for 1 mg of protein in the cell. The activity is presented as μmol H_2O_2 mg protein^{-1}.

Table 3.4: Determination of catalase activity

Chemicals	**Sample (ml)**	**Blank (ml)**
CAT reaction mixture	2.5	2.5
H_2SO_4	-	1.0
Sample extracts	0.05	0.05
Incubate for 10 min		
H_2SO_4	1.0	-
Distilled water	1.45	1.45
Read absorbance at 240 nm		

3.6 Statistical analysis

All experiments were performed in biological triplicates. The data were recorded and then analysed using student t-test and one-way analysis of variance (ANOVA) followed by Tukey's multiple range test at a significance level of 5 %.

4.0 Results and discussion

4.1 Initiation of embryogenic callus

A total of 850 male inflorescences of *Musa acuminata* cv. 'Berangan' (AAA) cultured on M1 medium were used to induce somatic embryogenesis. Male inflorescence has been considered to be one of the most suitable explants for the establishment of banana cell suspension cultures (Darvari *et al*., 2012; Kulkarni and Bapat, 2013; Elayabalan *et al*., 2013). Besides male inflorescence, other explants such as meristem and scalps have also been reported (Subramanyam *et al*., 2011; Divakaran and Nair, 2011).

After two weeks of culture, the base of flower clusters turned brown and swollen, indicating the formation of callus and somatic embryos. This browning effect might be due to the oxidation of phenolic compounds released by the injured tissues and cell division activities (Nisyawati and Kariyana, 2013). Exudation of a large quantity of phenolic compounds into medium might be toxic to the cells and caused cell death (Ko *et al.*, 2009). Therefore, in this study, ascorbic acid was added as an antioxidant agent to reduce browning and to curtail the exudation of phenolic compounds by scavenging reactive oxygen species produced from the damaged explants (Titov *et al.*, 2006).

To initiate embryogenic tissue, M1 medium containing 4 mg l^{-1} 2,4-D, 1 mg l^{-1} IAA, and 1 mg l^{-1} NAA was used. Induction of somatic embryogenesis is usually restricted to certain responsive cells in the explant under influence of auxins or other plant growth regulators (Rai *et al.*, 2007). Auxins, such as 2,4-D, have been commonly used to promote cell dedifferentiation to form embryogenic tissue. It has been considered as one of the most effective auxins for the induction of somatic embryogenesis in many plant species. Auxins enhance the cell division and

simultaneously terminate the cell elongation (Mujib and Šamaj, 2006). Inclusion of 2,4-D in the medium has been found to activate endogenous IAA that could lead to the formation of somatic embryos (Thomas *et al.*, 2002).

Embryogenic callus was first observed under microscope after eight months of culture (Figure 4.1). In this study, only 1.17 % of explants were successfully formed whitish translucent somatic embryos on a mass of friable callus. This might be due to the slow growth and necrosis tendency of banana callus (Chong-Pérez *et al*., 2012). Besides, the formation of somatic embryogenesis in banana has also been reported to be genotype-, cultivar- and types of clones-dependent (Youssef *et al*., 2010). Similar findings have been reported by Escalant *et al.* (1994) and Chong-Pérez *et al*. (2012). Escalant *et al.* (1994) reported that less than 5 % of banana cv. 'Grande Naine' (AAA) explants was induced to form somatic embryo, whereas Chong-Pérez *et al.* (2012) reported that only 1.36 % of banana cv. 'Dwarf Cavendish' (AAA) explants responded after 5 to 6 months of culture.

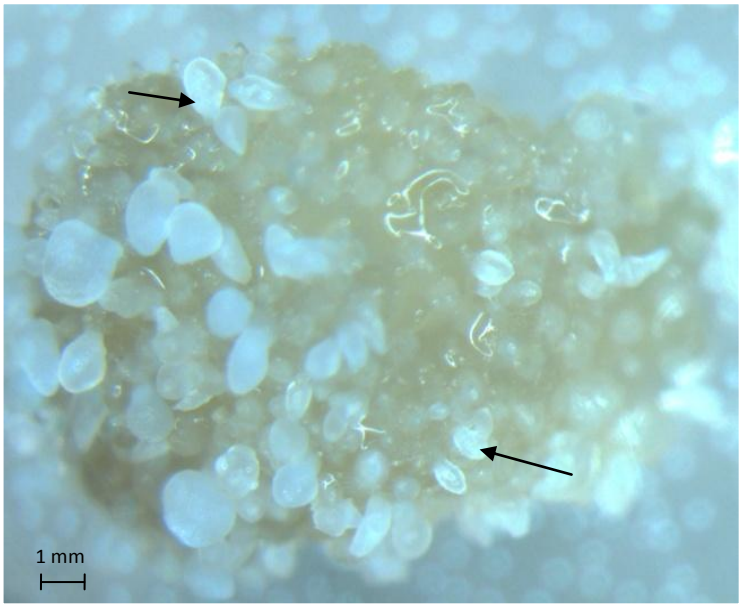

Figure 4.1: Translucent embryos (arrow) observed on friable callus of *Musa acuminata* cv. 'Berangan' after 8 months of culture on M1 medium.

In this study, the first 15 flower clusters closest to the dome were used to induce somatic embryogenesis as they are the youngest meristematic part which actively undergoes cell division. Age of explants has been considered as one of the important factors to establish tissue culture plantlets (Smith, 2012). It was observed that flower clusters at positions of 8 to 10 produced the highest percentage of somatic embryos (70 %) and no embryogenic callus were formed at positions 1 to 7, probably due to the high amount of active meristemic cells found at these positions (Figure 4.2). This was in accordance with the study carried out by Jalil *et al.* (2003), where more than 50 % of flower clusters at positions of 4 to 11 produced embryogenic callus. Of these, 18 % of the embryogenic calli were obtained from the flower cluster at position 9. Wei *et al.* (2005) found that floral clusters from positions 6 to 12 generated higher frequency of embryogenic callus compared to other positions.

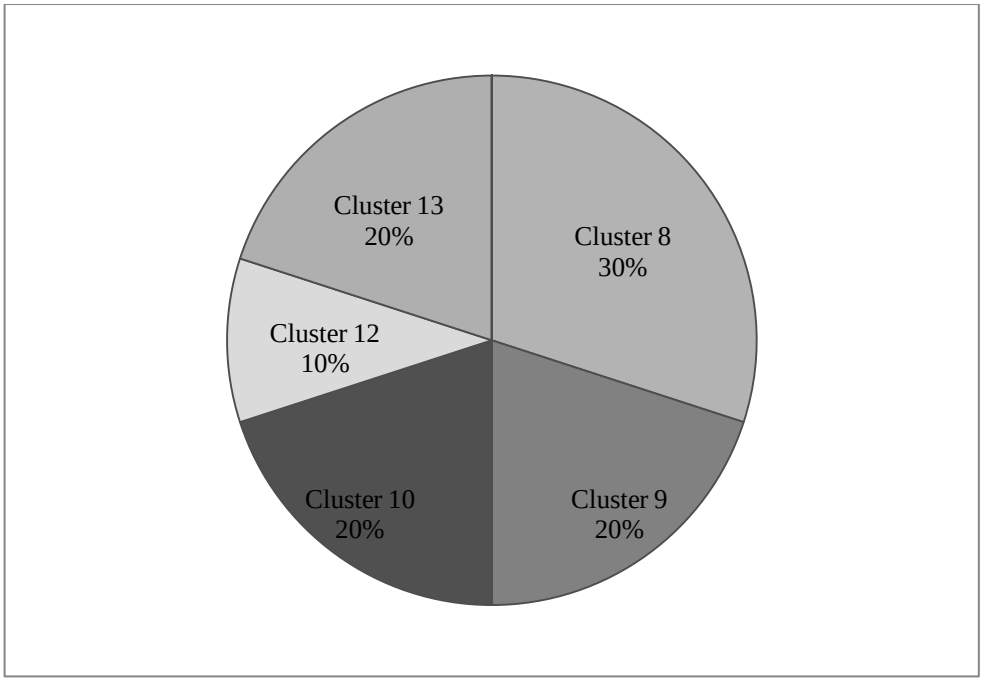

Figure 4.2: The formation of somatic embryo from different flower cluster positions of *Musa acuminata* cv. 'Berangan'. Clusters 1 to 7, 11, 14, and 15 did not produce any somatic embryos.

The generated embryogenic callus and somatic embryos were often heterogeneous. Only somatic embryos at an early stage and non-organised embryogenic cell clusters are able to give rise to embryogenic liquid culture (Strosse *et al.,* 2006). Therefore, it is important to select a good quality embryogenic complex for the establishment of cell suspension culture. Friable embryogenic complexes were found to be suitable for subsequent liquid cell suspension culture, whereas compact embryogenic calli were not suitable as they usually hard to disperse in liquid and large embryos tend to become necrotic (Tripathi *et al.*, 2012). Thus, regular observation on the induced callus is critical in order to determine the possibility of forming embryogenic callus.

The duration of forming embryogenic callus in *M. acuminata* cv. 'Berangan' (AAA) was longer (8 months) compared to other banana cultivars, such as 'Mas' (AA) and 'Grande Nain' (AAA) (5-6 months) (Côte *et al.*, 1996; Jalil *et al.*, 2003). This may be due to the cultivar differences, where banana cultivar AAA group was less responsive to somatic embryogenesis compared to AAB and ABB cultivar groups (Jalil *et al*., 2003). This finding was supported by Bakry *et al*. (2009), where banana cultivar with B genome was found to be more responsive compared to the A genome. In contrast, previous study demonstrated that AA genome group produced the highest frequency of embryo followed by AAB and AAA genomes (Assani *et al.*, 2002).

4.2 Establishment of cell suspension culture

Whitish embryogenic callus and translucent somatic embryos consisting of heterogeneous globules were transferred into modified M2 medium (Jalil *et al.*, 2003) for the establishment of cell suspension cultures (Figure 4.3).

Since liquid cultures could be easily contaminated by microorganisms or poisoned by phenolic compound excretion, thus, maintenance of sterility and elimination of phenolic exudates from the initial suspension culture is vital. To ensure a continuous nutrient supply and elimination of exudates excreted by the cells, the established cell suspension cultures in this study were subcultured by replacing half of the medium with fresh medium every 10 days. Big aggregates were also removed through this repeatedly subculture since starch tends to accumulate on their outer cells that could lead to the production of polyphenols.

After two months of culture, homogeneous, yellowish and fine embryogenic cell suspension cultures, which are an accepted embryogenic characteristics of banana cell suspension (Ganapathi *et al.*, 2001; Jalil *et al.*, 2003), were obtained after repeated sieving through a 425 μm metal mesh. Similar observation was reported by Ganapathi *et al.* (2001). Cell suspensioned would loose its embyogenic potential if the amount of embryognic clusters is lower than non-embryogenic clusters. Thus, sieving should be carried out from time-to-time to eliminate big cell clusters in the liquid suspension.

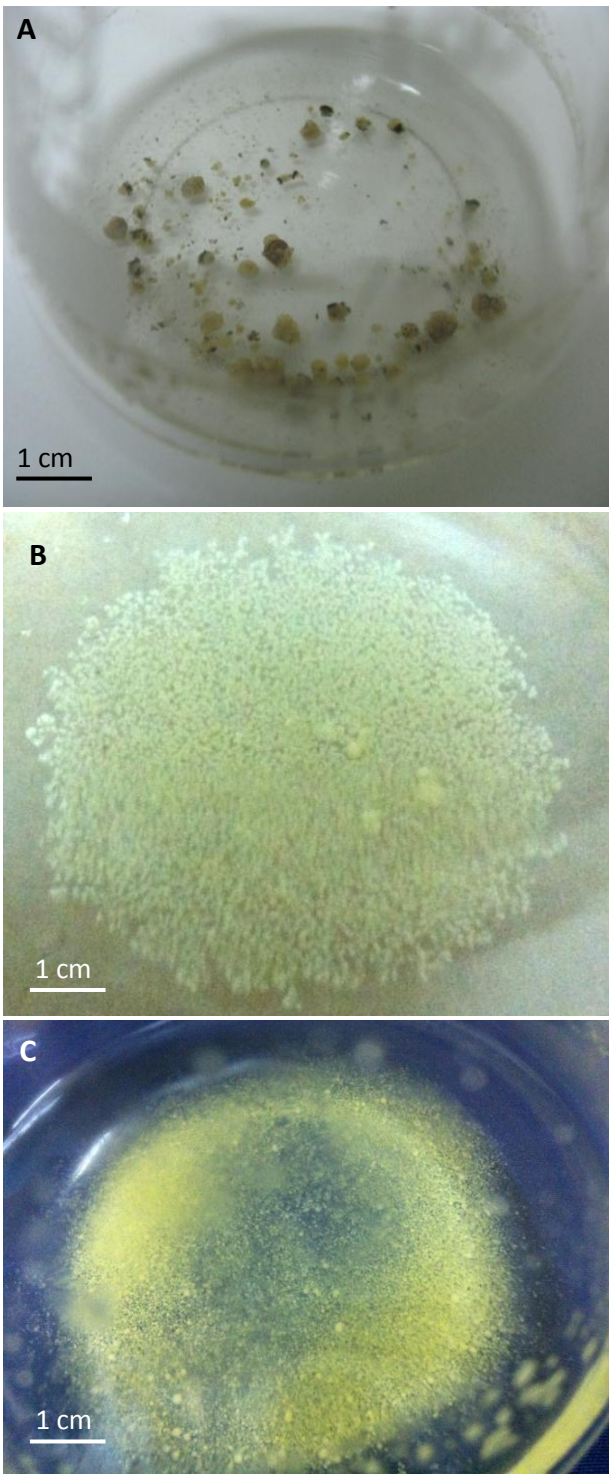

Figure 4.3: Establishment of embryogenic cell suspension in M2 medium. (A) one week old culture showing heterogeneous cells (B) one month old culture with a mixture of yellowish cells and cell clumps after sieving (C) two months old culture showing homogeneous, yellowish and fine embryogenic cells.

4.3 Optimisation of cell growth parameters in shake flasks

It is crucial to optimise key parameters in cell suspension cultures, such as oxygen level, sucrose concentration, medium content and inoculum density, which can greatly affect the production of biomass and secondary metabolites (Cui *et al.*, 2011). Hence, selected physical and chemical conditions were optimised using the previous established homogeneous cells of *M. acuminata* cv. 'Berangan' (AAA).

4.3.1 Effects of sucrose concentration

Sucrose is the most common carbohydrate found in phloem sap. It has been extensively used in plant cell cultures to provide energy and to maintain osmotic pressure in the cells (Wu and Ho, 1999). It also acts as signalling molecules for plant growth and development, stress resistance and metabolism (Moghaddam and Van de Ende, 2012). Sucrose and other sugars have been known to modulate the expression of many genes related to plants metabolism, respiration, defence system and photosynthesis (Jang *et al.*, 1997). Therefore, sucrose concentration plays an important role in *in vitro* cell growth, root formation and production of secondary metabolites (Cui *et al.*, 2010; Ferri *et al.*, 2011).

In this study, the responses of cell suspension cultures grow under three different concentrations of sucrose were tested. The results showed that MS medium containing 20 g l^{-1} produced yellowish cells compared to browning cells from medium containing 10 and 30 g l^{-1} sucrose (Figure 4.4). To further determine the optimum concentration of sucrose, a narrow range of sucrose concentrations (15 to 35 g l^{-1}) were tested.

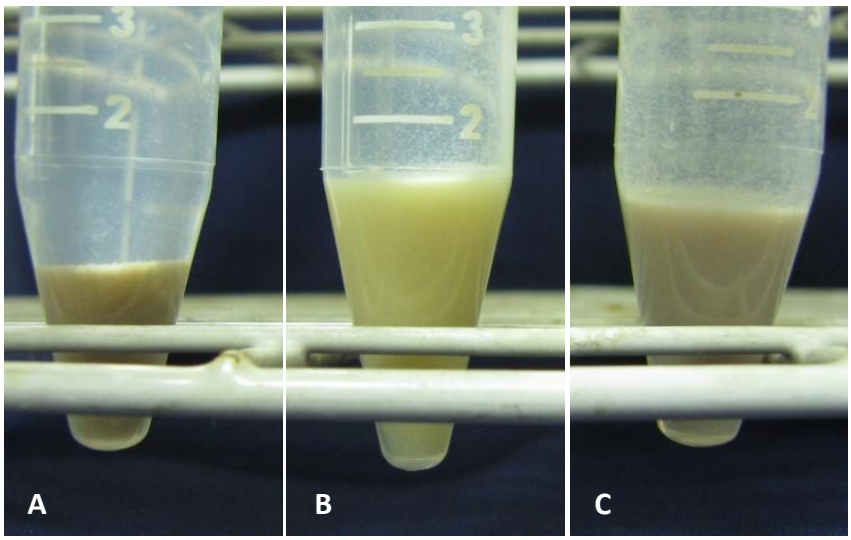

Figure 4.4: Effects of different concentrations of sucrose on cells cultured in M2 medium at day 14. (A) Browning cells in 10 g l^{-1} sucrose showing no growth (B) healthy and yellowish cells cultured in 20 g l^{-1} showing good growth and (C) Browning cells in 30 g l^{-1} sucrose showing moderate growth.

Medium containing 20 g l^{-1} sucrose produced the highest settled cell volume (SCV) of yellowish homogeneous cell suspensions, whereas less vigorously growing brownish cells were observed when cultured in MS medium containing sucrose concentrations either less than 20 g l^{-1} or more than 25 g l^{-1} (Figure 4.5). Cells cultured in MS medium supplemented with 10 g l^{-1} sucrose exhibited browning and slow growth with an increased rate of less than 0.1 ml SCV after 5 days of culture. This might be due to inadequate supply of carbohydrate source to the cells for growing. Similar observation has been found in medium containing 30 and 35 g l^{-1} sucrose. Cells cultured in both media exhibited browning effect after 10 days and died at the end of the culture cycle (14 days after culture). This might be due to the osmotic stress caused by high amount of sucrose.

High sucrose concentration in the medium creates high osmotic pressure that could retard the development of cultured cells (Wu *et al.*, 2006) and cause the cells to lose water. This negative effect may damage the cells and eventually lead to cell death.

This was in agreement with the study carried out by Cui *et al*. (2010), where the medium containing 30 g l^{-1} sucrose was found to produce maximum biomass in *Hypericum perforatum* L., whereas higher concentrations of sucrose (50, 70 and 90 g l^{-1}) inhibited biomass accumulation and root growth. Therefore, determination of optimum sucrose concentration is essential for cell growth.

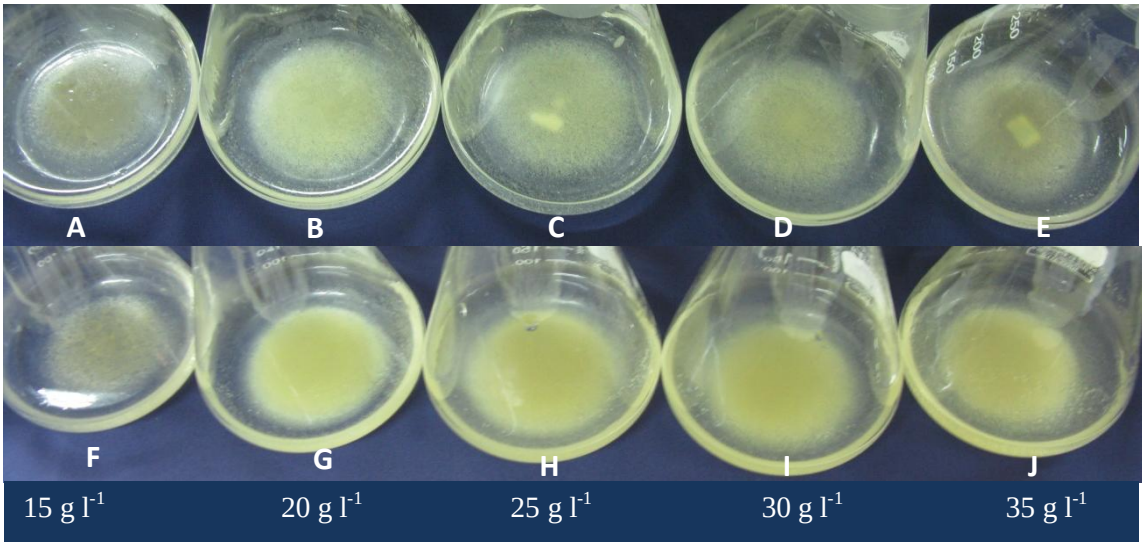

Figure 4.5: Comparative observations on morphology and growth of cell suspensions of *Musa acuminata* cv. 'Berangan' cultured in different sucrose concentrations on day 5 (A to E) and 10 (F to J). A and F: necrotic brown cells lacking growth in 15 g l^{-1} sucrose. B and G: healthy and yellowish cells showing good growth in 20 g l^{-1} sucrose. C and H: healthy and yellowish cells showing moderate growth in 25 g l^{-1} sucrose. D and I: brownish yellow cells showing slow growth in 30 g l^{-1} sucrose. E and J: Yellowish brown cells showing slow growth in 35 g l^{-1} sucrose.

4.3.2 Effects of initial inoculum density

Initial inoculum density has been known to be one of the important factors affecting cell growth (Gorret *et al*., 2004), biomass and metabolite accumulation (Zhong and Yoshida, 1995), and hairy root growth (Dehghan *et al*., 2012). Therefore, the effect of initial inoculum densities on cell growth was investigated.

It is shown that 1.0 ml SCV in 50 ml (2 %) culture medium gave the highest specific growth rate (5.3×10^{-2} / day) with vigorously growing cells. In contrast, relatively higher amount of brown cells was observed at inoculum level of 0.5 ml SCV. This might be due to longer lag phase or programmed cell death by the plant itself (McCabe and Leaver, 2000). Higher initial SCV (1.5 and 2.0 ml) produced less number of vigorously growing cells compared to 1.0 ml SCV, probably due to the increased amount of cells that competing for the limited nutrients. Similar results were found in the cell culture of *Withania somnifera* (Nagella and Murthy, 2010). Low amount of inoculum density (10 g l^{-1}) was found to yield maximum biomass compared to 20 g l^{-1} initial inoculum density. Loc and Nhat (2013) found that the highest cell growth index (4.78) was recorded in *Centella* cell culture when initial cells of 30 g were used compared to 100 g cells (3.03).

4.3.3 Effect of optimised sucrose concentration and initial inoculum density

Combination of different sucrose concentrations and initial inoculum densities were investigated to determine the optimum culture condition for cell suspension cultures. The results indicated that M2 medium containing 20 g l^{-1} sucrose with 1 ml initial inoculum density was suitable for cell suspension cultured in 250 ml shake flask as it produced the highest specific growth rate (5.7×10^{-2} / day) (Figure 4.6). This finding concurred with the results obtained in Sections 4.3.1.1 and 4.3.1.2.

Although sucrose with concentrations more than 25 g l^{-1} revealed similar specific growth rates, however, the cells exhibited browning symptom after 10 days of culture (Figure 4.6). This browning effect may affect the regeneration ability of embryogenic cells.

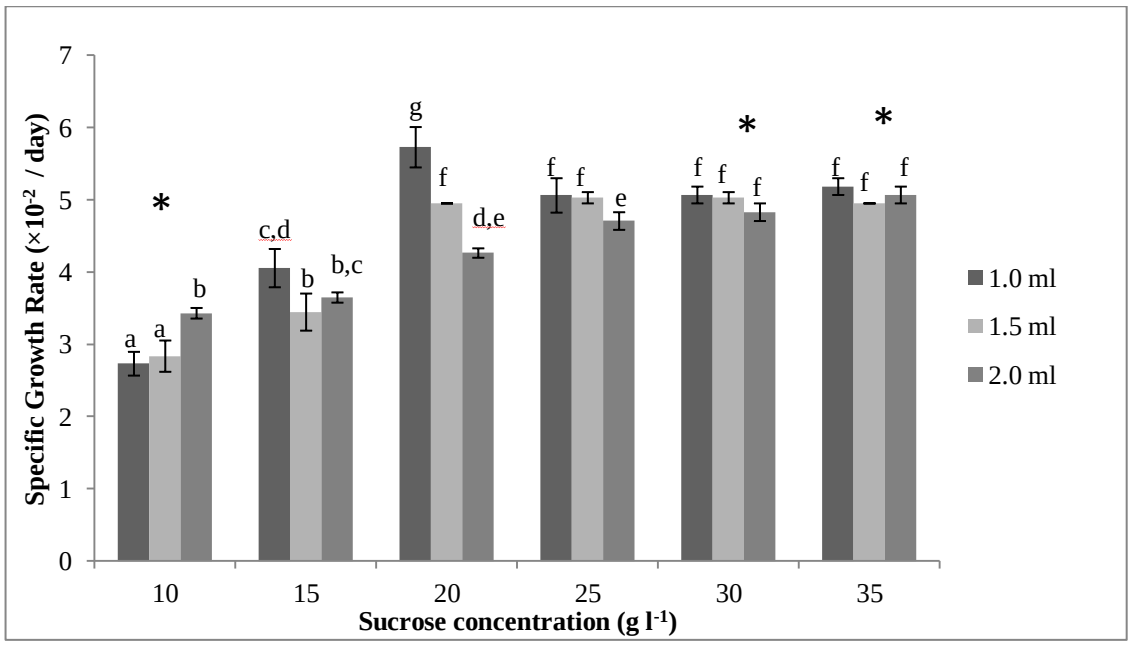

Figure 4.6: Effects of sucrose and initial inoculum density on cell growth. Results represent mean ± standard deviation of three replicated experiments after 14 days of culture. Different letters indicate a significant difference at $p < 0.05$. (* = cell browning after 14 days of culture).

4.3.4 Effects of vessel size

The cell suspension cultures were grown in three sizes of shake flasks, 250, 500 and 1000 ml, with an initial inoculum density of 1 ml. 1 ml initial inoculum density was found to be an optimal ratio for cell growth. However, no significant difference between the specific growth rate when doubled the flask volume. However, 4-fold increase in flask volume showed a significant difference in specific growth rate compared to shake flask (Table 4.1). It is noted that the cells grown in 1000 ml shake flask turned brown after 7 days of culture, whereas cells cultured in 250 and 500 ml shake flasks was found to be healthy. Kieran *et al.* (1997) indicated that the duration, intensity and physiological conditions are the main factors leading to the positive (e.g. increase cell biomass or secondary metabolite) or negative (e.g. sheared cells) responses.

In this study, we found that with the same inoculum: media ratio, specific growth rate of cells in different sizes of shake flask is significantly different. Specific growth rate of

cells cultured in 250 ml shake flask was comparable ($5.18 \pm 0.12 \times 10^{-2}$ / day) to 500 ml ($4.89 \pm 0.06 \times 10^{-2}$ / day) but significantly higher ($p < 0.05$) than 1000 ml shake flasks ($4.52 \pm 0.17 \times 10^{-2}$ / day). This may be due to the cell-to-cell interaction in the shake flasks tested where volatiles excreted during culture positively stimulated cell growth. Volatiles such as ethylene have been shown to promote cell growth (Pierik *et al*., 2006).

Table 4.1: Specific growth rate of cell suspension of *Musa acuminata* cv. 'Berangan' in different size of shake flasks after 14 days of culture

Volume of shake flask (ml)	**Total volume (ml)**	**SCV: medium ratio**	**Specific growth rate (μ_{max}) ($\times 10^{-2}$ / day)**	**Observation**
250	50	1 : 50	5.18 ± 0.12^{a}	Yellowish, healthy
500	100	2 : 100 (1 : 50)	4.89 ± 0.06^{ab}	Yellowish, healthy
1000	200	4 : 200 (1 : 50)	4.52 ± 0.17^{b}	Brownish

Results represent mean ± standard error of triplicate experiments. Different letters indicate a significant difference at $p < 0.05$

4.3.5 Effects of photoperiod

Effect of photoperiods on cell growth was also investigated. Differences between 24 h photoperiod and 16/8 h photoperiod cultivations were not significant in term of specific growth rates (Table 4.2). The cells cultured under both photoperiods appeared to be normal and healthy. Nevertheless, the size of cells under 16/8 h photoperiod appeared to be relatively larger compared to 24 h photoperiod (Figure 4.7). This observation suggested that the longer exposure time of high intensity light source might affect the cell development. Since the big cells tend to form cell clumps, thus, cultures obtained from 24 h photoperiod were selected for the subsequent experiments.

Table 4.2: Effect of photoperiod on cell suspension growth of *Musa acuminata* cv. 'Berangan' after 2 weeks of culture in shake flasks

Photoperiod (light/dark) (h)	**Specific growth rate (μ_{max}) ($\times 10^{-2}$ / day)**
16/8	5.51 ± 0.29^{a}
24/0	5.84 ± 0.21^{a}

Results represent mean ± standard error of triplicate experiments. Different letters indicate a significant difference at $p < 0.05$

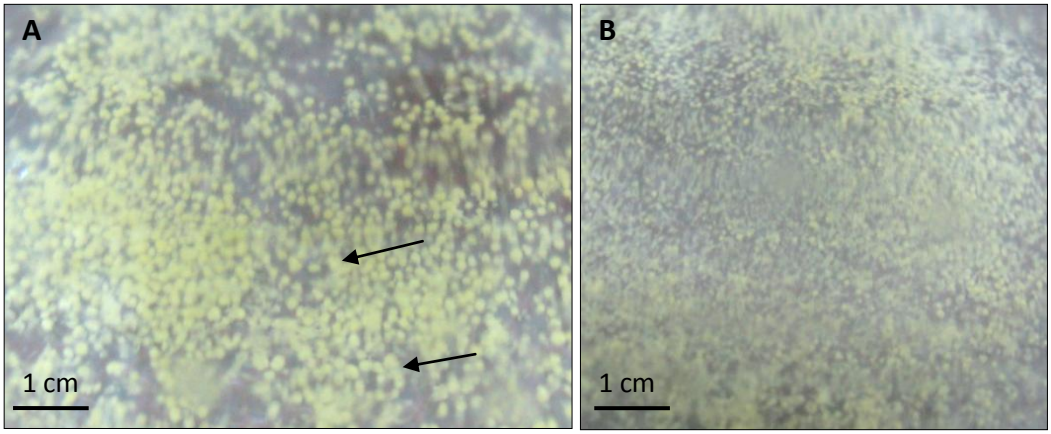

Figure 4.7: Morphology of cell suspension cultures of *Musa acuminata* cv. 'Berangan' under different photoperiods. (A) Cell clumps formation (arrow) observed in 18/6 h photoperiod (B) Fine homogeneous cells observed in 24 h photoperiod.

4.3.6 Growth curve and pH curve of cell suspension culture

The growth of cell suspension was recorded every two days by measuring SCV of the cells. It was found that the cells showed a lag phase for the first four days. The growth rate gradually increased and reached the highest specific growth rate at 5×10^{-2} / day after two weeks of culture (Figure 4.8). The cell population approximately doubled after 14 days. This was in agreement with the study carried out by Cote *et al.* (1996), where a 2- to 5- fold growth was recorded within 30 days in 'Grande Naine' cell suspension culture. Accumulation of waste and depletion of nutrient in the media may cause cells to turn brown and eventually lead to cell death. Hence, in this study, the cell suspension cultures were sub-cultured or harvested at 14th day before the cells reached its stationary phase.

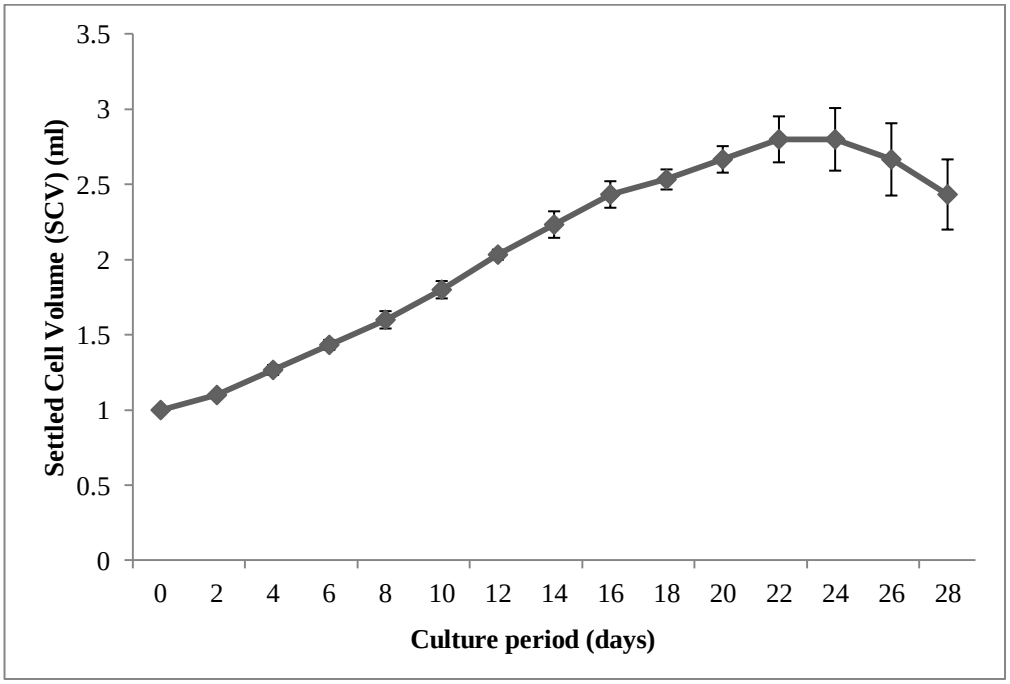

Figure 4.8: Growth curve of *Musa acuminata* cv. 'Berangan' cell suspension culture in shake flasks. Error bars correspond to standard deviation of triplicate measurements.

The pH of media in the shake flasks decreased steeply from 5.7 to 3.8 within the first seven days (Figure 4.9). The sudden drop of the pH might be due to the uptake of NH_4^+ and NO_3^- by plant cells in the culture medium (Chung *et al.*, 2006). During the first four days, pH decreased drastically for both 250 and 500 ml flasks at a rate of Δ1.5 and Δ1.8 pH unit, respectively. In comparison, pH of cell-free medium (control) recorded insignificant decrease at approximately Δ0.8 over 14 days (Figure 4.9). It is also noted that the pH for all media remained unchanged after day 7. The maximum growth of cell suspension cultures was observed on day 14 of the cultivation for both 250 and 500 ml shake flasks.

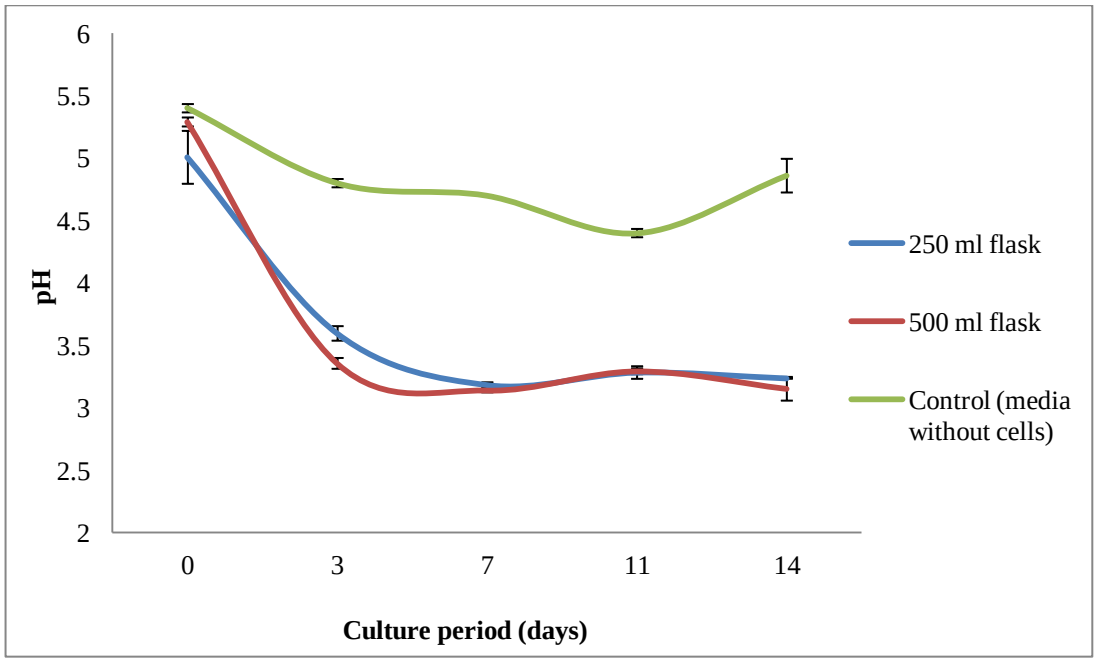

Figure 4.9: Effect of pH on *Musa acuminata* cv. 'Berangan' cell suspension culture. The pH of media in the shake flasks decreased steeply within the first seven days of culture. Error bars correspond to standard deviation of triplicate measurements.

4.4 Optimisation of cell growth in balloon type bubble column bioreactor (BTBCB)

Productivity of cells usually decreased when mass propagated in a bioreactor, due to the different hydrodynamic conditions of a bioreactor arising from different shear stress, oxygen supply and gas composition compared to conventional shake flask (Qian *et al.*, 2005). Clogging in the vent of column bioreactor due to excessive foaming by cell suspension cultures is a common problem (Paek *et al*., 2001). To meet this challenge, a modified balloon type bubble column bioreactor (BTBCB), designed by Paek *et al.* (2005), was chosen to mass-produce *M. acuminata* cell suspension cultures. This type of bioreactor could help to reduce the foaming by implementing a wider surface area at the top of bioreactor above the concentric tube that forms the base of the bioreactor (Figure 4.10). Large scale plant production using BTBCB has been reported in *Panax ginseng*, orchids and other valuable plants (Paek *et al.*, 2005).

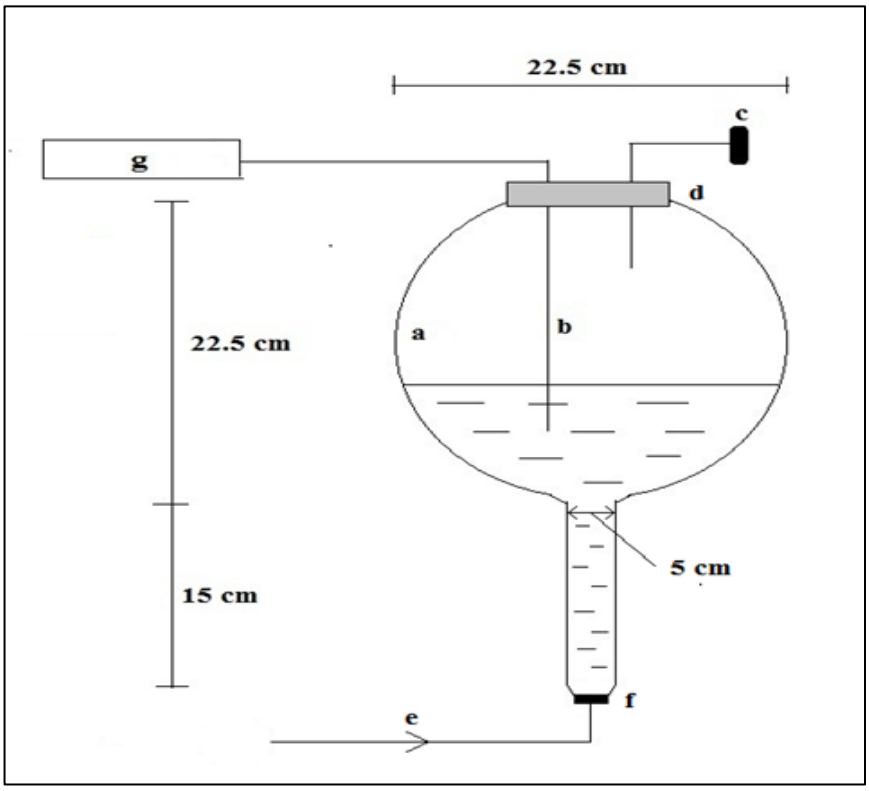

Figure 4.10: Schematic diagram of a 5 l balloon type bubble column bioreactor (BTBCB). (a) BTBCB, (b) pH meter, (c) filter vent, (d) silicon cap, (e) filtered air inlet, (f) automated controller, and (g) acid and alkali controllers.

In bioreactor, the pH of the medium decreased to 3.8 at day 5 of culture with a rate of Δ0.38 pH unit / day. Similar results were recorded in the shake flask cultures. The cell culture in the BTBCB showed higher specific growth rate (6.5×10^{-2} / day) than shake flask cultures (5.7×10^{-2} / day) (Figure 4.11).

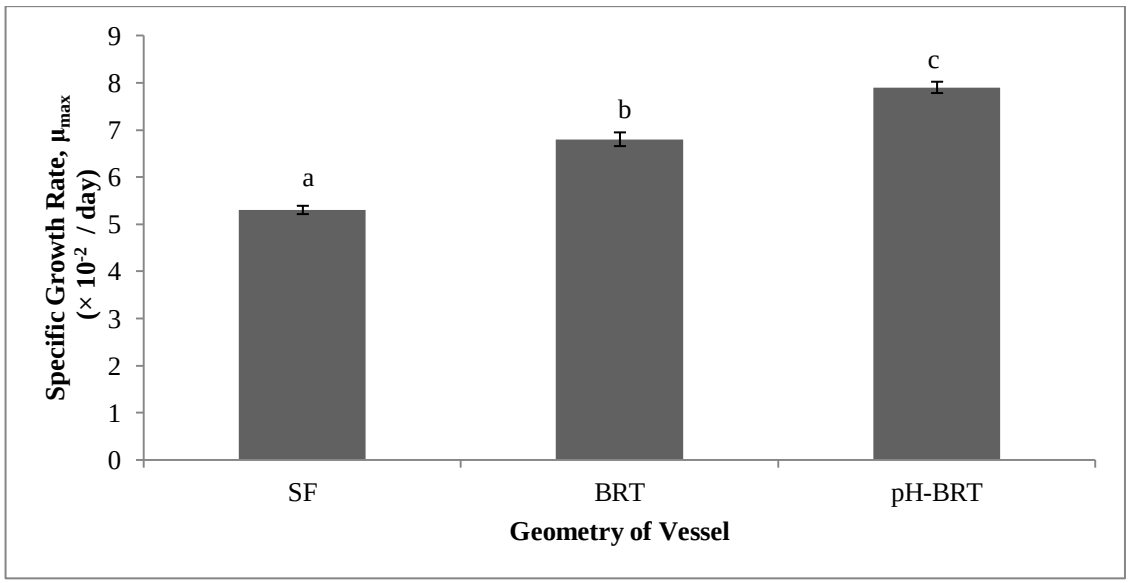

Figure 4.11 : Specific growth rate of *Musa acuminata* cv. 'Berangan' in shake flask (SF), non-pH-controlled BTBCB (BRT) and pH-controlled BTBCB (pH-BRT) over 14 days of culture. Results represent mean ± standard error of triplicate experiments. Different letters indicate a significant difference at $p < 0.05$.

The growth profile of cell suspension in shake flask and BTBCB are shown in Figure 4.12. Differences in gaseous component between the cultivation systems has slight changed the cell growth. In BTBCB, external sterile gas pumped into the liquid medium using a compressor led to better mixing conditions for the cells, particularly in dissolving essential gaseous nutrient such as oxygen (O_2). Orbital shaking as performed on the shake flask culture, however, was unable to allow such mixing condition, especially for sparingly soluble nutrient like O_2. O_2 only merely supply to the culture *via* headspace gas entrainment due to the circular motion of the liquid. Fluid mixing through forced air also helped active removal of gaseous by-products, such as CO_2, in the media through the vent of the BTBCB (Zhong *et al.*, 2001). Gases, such as CO_2 and

ethylene, have been considered as one of the factors affecting cell growth or synthesis of secondary metabolites in plant tissue culture. All these conditions may lead to different cell growth. In this study, bioreactor was more favourable for the growth of banana cell suspension (Figure 4.11).

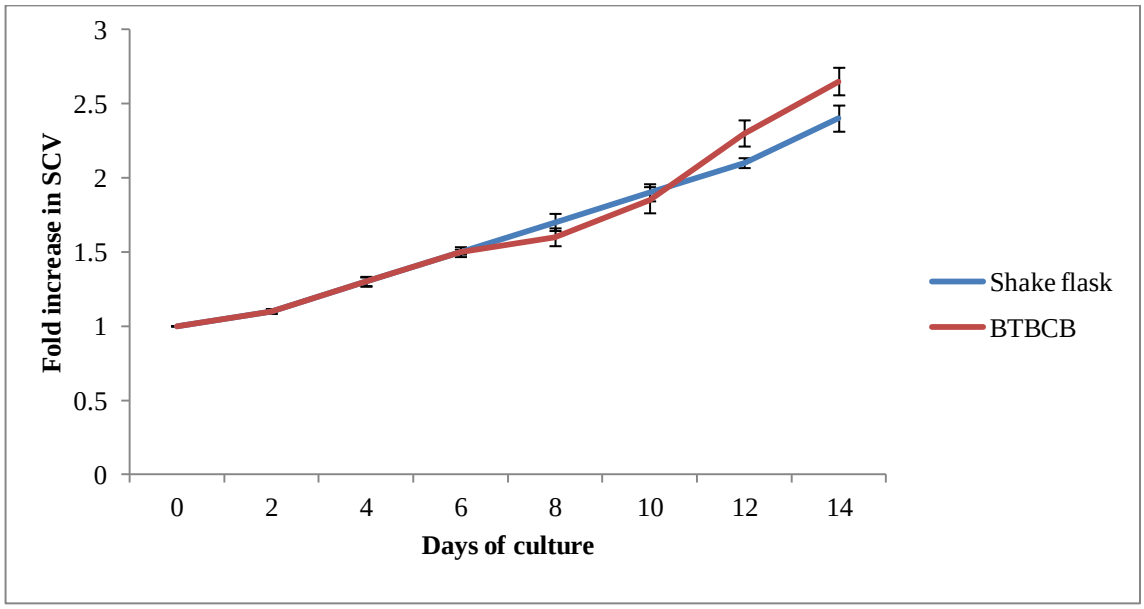

Figure 4.12: Growth profile of *Musa acuminata* cv. 'Berangan' in shake flask and non-pH-controlled BTBCB over 14 days of culture. Results represent mean ± standard error of triplicate experiments.

Three different airflow rates were tested for the cultivation of cell suspension culture in BTBCB culture. At 50 cc min^{-1} (0.05 vvm) flow rate, some of the cells were found to be settled at the bottom of the balloon (Figure 4.12). These cells turned brown at the end of 14 days of culture. On the other hand, healthy cells with higher specific growth rate was found after 14 days of culture when cultivated in a bioreactor at 100 cc min^{-1} (0.1 vvm) flow rate (Figure 4.12). However, the cells became brown at day 5 of culture and tend to stick against the BTBCB's wall and pH probe under 200 cc min^{-1} (0.2 vvm) flow rate. Serious forming was also observed when cells cultured at this flow rate (Figure 4.13).

Foaming is normally correlated to extracellular protein concentration or aeration rate of the bioreactor (Wongasmuth and Doran, 1994). How extracellular protein or medium components affect the potential and stability of foaming in the bioreactor culture is still not comprehensive understood. In some studies, antifoaming agent was used to control foaming but this may result in reduced productivity of the system (Wongasmuth and Doran, 1994). According to Zhong *et al.* (1992), excessive aeration rate can lead to a serious foaming problem in plant cell suspension culture in bioreactor. Thus, in this study, we found that airflow rate at 100 cc min^{-1} (0.1 vvm) was suitable for the culture of banana cell suspension.

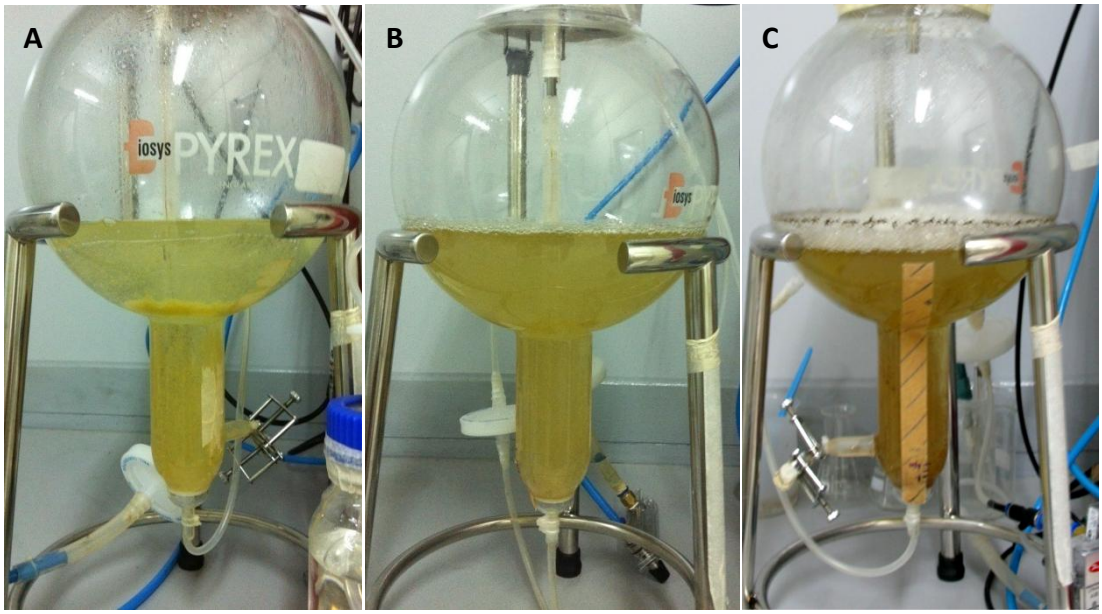

Figure 4.13: The effects of airflow rate of (A) 50, (B) 100, and (C) 200 cc min^{-1} on cell suspension cultures at day 5.

4.4.1 pH-controlled balloon type bubble column bioreactor (pH-controlled BTBCB)

In another experiment, different culture vessels (shake flasks, non-pH-controlled BTBCB and pH-controlled BTBCB) used for culturing banana cells were compared. The specifications for the culture vessels are shown in Table 4.3. After 14 days of culture, the cells cultured in pH-controlled BTBCB showed higher specific growth rate (7.9×10^{-2} / day) compared to non-pH-controlled BTBCB (6.5×10^{-2} / day) and shake flasks (5.7×10^{-2} / day) (Figure 4.11). The highest growth yield was recorded in pH-controlled BTBCB (3.01), followed by non-pH-controlled BTBCB (2.53) and shake flask (2.23). The fold increase of banana cell suspension cultures in shake flask, non-pH-controlled BTBCB and pH-controlled BTBCB are shown in Figure 4.14.

Medium with pH 5.7 has been widely used in culturing *in vitro* plantlets. Under this pH condition, mineral salts are more easily dissolved in the medium and absorbed by plant cells (Murashige and Skoog, 1962). This underlined the importance of controlling pH in cell suspension cultures as the nutrients uptake is greatly affected by the pH environment (George *et al.*, 2008). For instance, strong acidic condition might affect the proliferation of cell clusters (Chung *et al.*, 2006).

Table 4.3: Comparison of different culture system for *Musa acuminata* cv. 'Berangan' cell suspension cultures

	Shake flask	**Non-pH-controlled BTBCB**	**pH-controlled BTBCB**
Vessel size	250 ml	5000 ml	5000 ml
Media volume	50	1000	1000
Initial cell volume (Settled cell volume, SCV)	1	20	20
Air supply	Agitation by shaker	Air flow meter, 100 cc min^{-1}	Air flow meter, 100 cc min^{-1}
pH	Non-controlled	Non–controlled	Controlled at 5.7

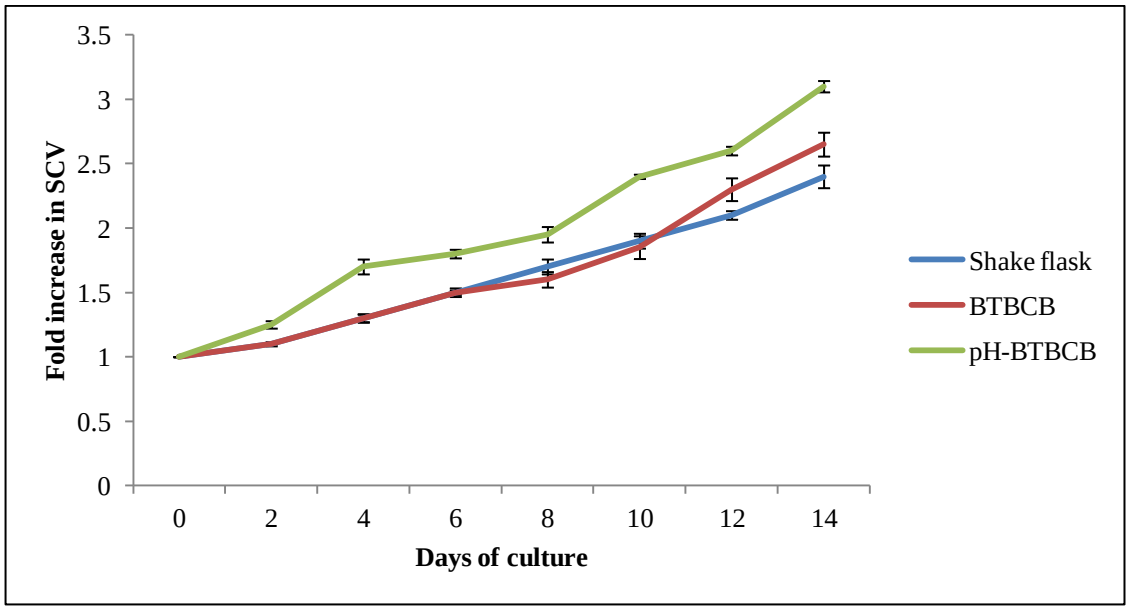

Figure 4.14: Fold increase in SCV of *Musa acuminata* cv. 'Berangan' in shake flask (SF), non-pH-controlled in BTBCB (BRT) and pH-controlled BTBCB (pH-BRT) over 14 days of culture. Results represent mean ± standard error of triplicate experiments. Different letters indicate a significant difference at $p < 0.05$.

4.5 Embryo development and regeneration

To compare the development and regeneration ability of cell suspension cultures obtained from culture vessels tested, the cell suspension cultures were harvested and transferred to development medium (M3) in dark condition. Cells remained undifferentiated when transferred onto filter paper on M3 solid medium and started to differentiate and reached maturation stage after 2 weeks of culture in the development medium. After one month, translucent spheres and torpedo shaped embryos were observed under microscope (Figure 4.15). During development stage, starch which act as a storage reserve for carbohydrate was utilised to provide energy for the conversion process (Bhojwani and Soh, 2001).

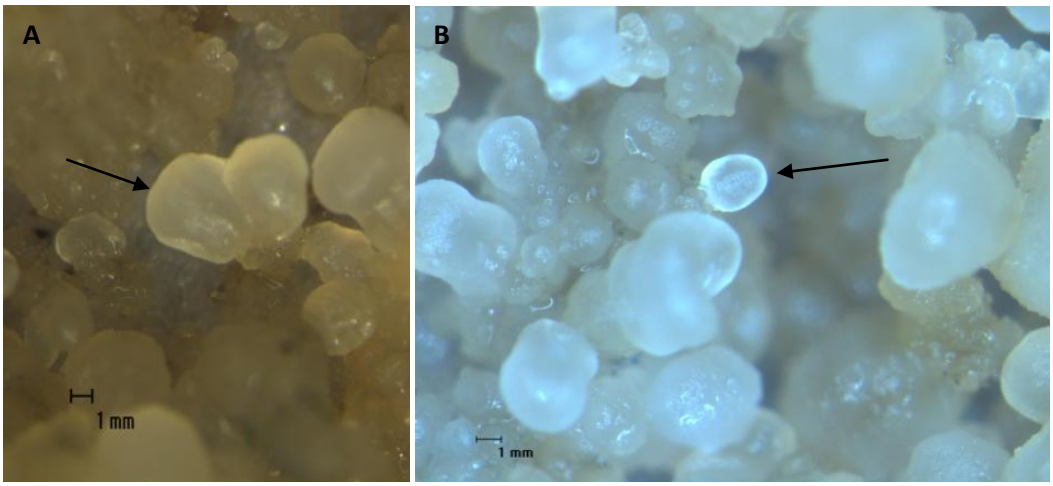

Figure 4.15: Shapes of somatic embryos observed on M3 medium after one month of culture. (A) Sphere shaped somatic embryo and (B) torpedo shape embryos.

Becker *et al.* (2000) reported that long culture period in M3 medium enabled cells to produce high frequency of germination. Similar finding has been reported by Stasolla and Yeung (2003). The authors indicated that embryos need longer incubation time to complete its maturation. After 6-8 weeks of culture, the developed embryos were transferred to M4 medium for the formation of chloroplast and shoots development (Figure 4.16).

Figure 4.16: Regenerated shoots on M4 medium after 8 weeks of culture.

For the shoot regeneration, MS medium supplemented with 8 mg l^{-1} BAP (M4) was used according to Jalil *et al.* (2003). After one month of culture, the generated plantlets were transferred to M4B medium containing lower concentration of BAP (4 mg l^{-1}) in order to minimise the occurrence of somaclonal variation and vitrification (Jalil, pers. comm., 2011).

The regeneration of banana cell suspension depends on the quality of cells. Highly regenerable cells can give rise to 10^4 to 10^5 somatic embryos per ml SCV. The frequency of regeneration (73.67 ± 4.48 %) obtained from the shake flasks was comparable to those from the pH-controlled bioreactor (62.33 ± 4.26 %) (Figure 4.17). From the regenerants obtained, 85.57 % were normal plantlets with respect to morphological characteristic compared to donor plant from the pH-controlled bioreactor (Table 4.4). Abnormal plantlets such as stunted growth plantlets and vernalised leaves were also observed (Figure 4.18). Rooting with M5 medium enabled 100 % individual plantlets to produce roots. Acclimatisation of plantlets under netted structure was performed after one month of rooting.

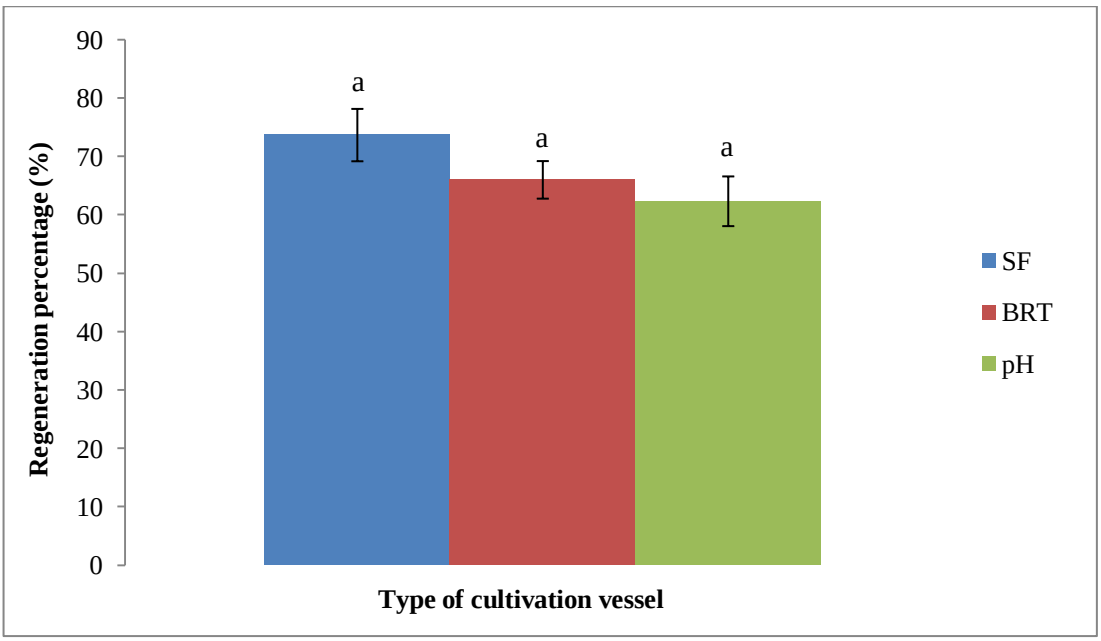

Figure 4.17: Percentage of regeneration for banana cell suspension in different cultivation vessels. SF: Shake flask, BRT: non-pH-controlled BTBCB, pH: pH-controlled BTBCB.

Table 4.4: Comparison on number of plantlets and regeneration percentage on different vessels for banana cell suspension cultures

Growth vessel	Developed embryos (%)	Number of shoots developed per 100 developed embryo	Number of plantlets per ml (SCV)	Normal plantlets (%)
SF	73.67 ±4.48[a]	166.0 ± 14.5[a]	8875 ±450[a]	83.33 ±2.38[a]
BRT	66.00 ±3.21[a]	140.3 ± 10.6[a]	8300 ±752[a]	80.44 ±0.58[a]
pH-BRT	62.33 ±4.26[a]	133.7 ± 12.9[a]	7825 ±447[a]	85.57 ±1.02[a]

Results represent mean ± standard error of triplicate experiments. Different letters indicate a significant difference at $p < 0.05$

Figure 4.18: Plantlets observed during regeneration stage. (A) Abnormal plantlets including vernalised and dwarf plantlets (B) Normal plantlet.

4.6 Protein estimation and stress induced enzyme activities

The protein content of the cells cultured in non-pH controlled BTBCB was the highest at 0.16 ± 0.002 % compared to shake flask and pH-controlled BTBCB (Table 4.5).

Table 4.5: The endogenous protein, H_2O_2 and stress induced enzyme activities of the cells grown in shake flask and bioreactors

Growth vessel	Percentage protein g^{-1} FW (%)	H_2O_2 (µmol g^{-1} FW)	CAT (µmol H_2O_2 mg^{-1} protein)	SOD (Unit 100 mg^{-1} FW min^{-1})
SF	0.11 ± 0.001	0.17 ± 0.01[a]	3.2 ± 0.6[a]	7.6 ± 0.1[a]
BRT	0.16 ± 0.002	0.24 ± 0.02[b]	4.2 ± 0.7[a]	7.1 ± 0.2[a]
pH-BRT	0.08 ± 0.001	0.30 ± 0.01[c]	10.3 ± 1.9[b]	7.2 ± 0.2[a]

Results represent mean ± standard error of triplicate experiments. Different letters indicate a significant difference at $p < 0.05$

Plants naturally develop antioxidant systems based on enzymes and non-enzymes mechanism to protect themselves against oxidative stress. Oxidative stress could lead to the production of ROS, such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^-), which also acts as a signal for defence reactions in plants (Mittler *et al.*, 2002). ROS may cause damaged in DNA, inactive enzymes and lipid peroxidation. To avoid oxidative stress, enzymes such as malonaldehyde (MDA), superoxide dismutase (SOD) and peroxidase (POD) are very important role in countering the detrimental effects of the ROS.

In this study, selected antioxidant enzymes assays and endogenous H_2O_2 levels were investigated in order to determine the effects of culture vessels on the cell physiology. Endogenous H_2O_2 concentration was found to be higher in cells cultivated in pH-controlled BTBCB (0.30 μmol g^{-1} FW), followed by uncontrolled-pH BTBCB (0.24 μmol g^{-1} FW) and shake flask (0.17 μmol g^{-1} FW).

In this study, higher H_2O_2 level was observed for cells cultivated in bioreactor maintained at constant pH 5.7 compared to shake flask and non-pH controlled bioreactor. This observation indicated that the cells were more stress when cultivated in bioreactor at constant pH 5.7. H_2O_2 acts as a signaling molecule to activate plant defense system when cells under stress conditions. Therefore, cellular content of H_2O_2 usually reflects cellular damage resulted from oxidative stress (Ozden and Karaaslan, 2011). However, the effect of H_2O_2 in plants varied according to different conditions (Quan *et al.*, 2008). Quan *et al.* (2008) reported that H_2O_2 at an acceptable level may help in promoting plant growth and development.

It is interesting to note that despite the high level of H_2O_2 detected in the banana cells cultivated in pH-controlled bioreactor, the SOD activities of the systems tested were approximately similar (Table 4.5). Expression of SOD was not significantly influenced by the H_2O_2. SOD has been considered as the first line of defense system against ROS, by converting two superoxide anions into a hydrogen peroxide and an oxygen molecule.

CAT was found to have similar trend with H_2O_2, in which the highest CAT level (10.3 µmol H_2O_2 mg^{-1} protein) was detected in pH-controlled BTBCB, whereas the lowest (3.2 µmol H_2O_2 mg^{-1} protein) was recorded in shake flask. Similar findings have been also reported in other plant species (Meratan *et al.*, 2009; Abbasi *et al.*, 2011). In plants, H_2O_2 generated from mitochondrial electron transport, β-oxidation of the fatty acids, and in photorespiratory oxidation are converted by CAT into H_2O and O_2 (Sharma *et al.*, 2012).

Despite the formation of ROS that might affect proliferation capacity of plant cells, data presented here showed that cells grown in pH-controlled BTBCB were able to produce a significant higher growth rate than non-pH-controlled bioreactor and shake flasks.

5.0 Conclusion

In this study, somatic embryos were successfully initiated from male inflorescence of *Musa acuminata* cv. 'Berangan' (AAA). Approximately 1.2 % of embryogenic callus was induced after 8 months of culture in M1 medium. Of these, 70 % of the flower clusters at positions 8 to 10 was able to produce somatic embryos, whereas no embryogenic callus was obtained from positions 1 to 7.

The embryogenic callus was used to initiate embryogenic cell suspension (ECS). Homogenous and yellowish fine cells were obtained after two months of culture. To produce the highest cell growth rate, several parameters were optimised. The results revealed that 1 ml initial inoculum density (settled cell volume) cultured in M2 medium containing 20 g l^{-1} sucrose was able to produce the highest specific growth rate ($5.7 \pm 0.28 \times 10^{-2}$ / day) over 14 days of culture.

The established ECS was then cultivated in a 5 l BTBCB without pH control. The specific growth rate of ECS increased to 6.5×10^{-2} / day compared to shake flask culture. The specific growth rate of cells was increased to 7.9×10^{-2} / day when cultivated in the pH-controlled BTBCB. The highest growth yield of culture was recorded in pH-controlled BTBCB (3.01), followed by non-pH-controlled BTBCB (2.53) and shake flask (2.23).

The cells obtained from all culture vessels were harvested and analysed for ROS content. The results showed that H_2O_2 and CAT activities detected in pH-controlled BTBCB were significantly higher than shake flask and non-pH-controlled BTBCB. This might be due to the activation of the cell defence system in response to stress. However, despite the high level of antioxidative enzyme, regeneration frequency of normal

plantlets from each culture vessels was not significant. The level of ROS in the cells probably did not affect the regeneration of plantlets.

In conclusion, pH-controlled BTBCB has a good potential for cultivating banana cell suspension cultures at relatively dense level compared to the traditional shake flasks. High frequency of normal plantlets obtained from banana cell suspension grown in pH-controlled BTBCB could be a promising approach for automated large scale banana plantlets production.

5.1 Future works

Male inflorescence is the most commonly used explant to induce embryogenic cell suspension in *Musa* species. However, low percentage of somatic embryo formation using this type of explants continues to be a significant limitation. Thus, it is important to improve the frequency of somatic embryogenesis using other explants or to optimise the culture conditions, such as media composition and plant growth regulators.

This study showed that banana cell suspension cultures could be cultivated in a pH-controlled bioreactor at relatively dense level and significant growth rate as compared to the traditional shake flasks. Optimum plant growth conditions can be easily achieved in a bioreactor by regulating various chemical and physical factors, including mixing, gaseous composition, efficient oxygen transfer, pH and hydrodynamic forces in bioreactors. However, more parameters such as O_2 content, temperature, and air volume are essential to further investigate the effect of bioreactor on cell growth and its potential to be used for automated large scale banana plantlets production.

The endogenous H_2O_2 content and antioxidative enzyme activities of the cells grown in shake flask and bioreactors were investigated in order to determine the cell stress level. More enzymatic assays, such as POD, MDA, and 1,1-diphenyl-2-picrylhydrazyl (DPPH), should be carried out to further investigate the interaction of ROS and cells under different culture vessels. This piece of work represents a fundamental for further research that will provide a comprehensive understanding on the cellular biochemical changes in different culture vessels and could be potentially served as a biomarker for an early detection of somaclonal variation.

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APPENDICES

Appendix A: Materials used

Appendix A1: Murashige & Skoog (MS) basal salt medium (1962)

Component	Concentration (mg l^{-1})
Macronutrient	
$CaCl_2.2H_2O$	440
NH_4NO_3	1650
KNO_3	1900
KH_2PO_4	170
$MgSO_4.7H_2O$	370
Micronutrient	
KI	0.083
$CoCl_2 \cdot 6H_2O$	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
Iron chelate	
$FeSO_4.7H_2O$	27.850
$Na_2EDTA.2H_2O$	37.250
Vitamins	
Glysine	2.000
Nicotinic acid	0.500
Pyridoxine	0.500
Thiamine HCl	0.100
Myo-inositol	0.100

Appendix A2: Dhed'a Vitamins (1991)

Components	Concentration (mg l^{-1})
Glycine	0.002
Nicotinic acid	0.005
Pyridoxine.HCl	0.005
Thiamine.HCl	0.005

APPENDIX B: Raw data

Appendix B1: Effect of different sucrose concentrations and initial inoculum densities

Sucrose concentration ($g\ l^{-1}$)	Initial inoculum density (ml SCV)	Specific growth rate ($\times 10^{-2}$ / day)
10	1.0	2.73 ± 0.16^{a}
	1.5	2.84 ± 0.22^{a}
	2.0	3.43 ± 0.07^{b}
15	1.0	$4.06 \pm 0.26^{c,d}$
	1.5	3.45 ± 0.26^{b}
	2.0	$3.65 \pm 0.07^{b,c}$
20	1.0	5.73 ± 0.28^{g}
	1.5	4.95 ± 0.00^{f}
	2.0	$4.26 \pm 0.07^{d,e}$
25	1.0	5.06 ± 0.24^{f}
	1.5	5.03 ± 0.08^{f}
	2.0	4.71 ± 0.12^{e}
30	1.0	4.83 ± 0.12^{f}
	1.5	5.03 ± 0.08^{f}
	2.0	4.83 ± 0.12^{f}
35	1.0	5.18 ± 0.12^{f}
	1.5	4.95 ± 0.00^{f}
	2.0	5.07 ± 0.12^{f}

Appendix B2: Effect of pH in different sizes of flasks and media

Day	0	3	7	11	14
Flask 250	5.00 ±0.21	3.60 ±0.06	3.18 ±0.02	3.28 ±0.05	3.24 ±0.00
Flask 500	5.29 ±0.04	3.36 ±0.04	3.14 ±0.02	3.29 ±0.02	3.15 ±0.09
Media without cells	5.33 ±0.03	4.77 ±0.03	4.7 ±0.00	4.43 ±0.03	4.65 ±0.13

Appendix B3: Growth profile of cell suspension cultures in shake flask (SF), non-pH-controlled BTBCB (BRT) and pH-controlled BTBCB (pH-BRT) over 14 days of culture

Day	0	2	4	6	8	10	12	14
ID								
SF	1.00 ±0.00	1.10 ±0.00	1.27 ±0.03	1.43 ±0.03	1.60 ±0.06	1.80 ±0.06	2.03 ±0.03	2.23 ±0.09
BRT	20.00 ±0.00	22.67 ±0.33	26.00 ±0.58	29.33 ±0.33	32.67 ±1.20	37.67 ±1.76	43.33 ±1.76	50.67 ±1.86
pH-BRT	20.00 ±0.00	25.00 ±0.58	32.00 ±1.15	36.67 ±0.67	40.67 ±1.20	48.67 ±0.33	53.33 ±0.67	60.33 ±0.88

APPENDIX C: Statistical analysis

Appendix C1: Effect of sucrose concentrations and initial inoculum densities on cell suspension culture of *Musa acuminata* cv. 'Berangan'

ANOVA

Inoculum

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	39.335	17	2.314	31.270	.000
Within Groups	2.664	36	.074		
Total	41.999	53			

Homogeneous subset

Tukey HSD

Sucrose Concentration	N	Subset for alpha = 0.05						
		1	2	3	4	5	6	7
1	3	2.7333						
7	3	2.8333	2.8333					
13	3	3.4333	3.4333	3.4333				
8	3	3.4467	3.4467	3.4467				
14	3		3.6500	3.6500				
2	3			4.0533	4.0533			
15	3			4.2633	4.2633	4.2633		
16	3				4.7033	4.7033	4.7033	
17	3				4.8267	4.8267	4.8267	
9	3					4.9500	4.9500	4.9500
12	3					4.9500	4.9500	4.9500
10	3					5.0300	5.0300	5.0300
11	3					5.0300	5.0300	5.0300
4	3					5.0600	5.0600	5.0600
5	3					5.0667	5.0667	5.0667
18	3					5.0667	5.0667	5.0667
6	3						5.1833	5.1833
3	3							5.7267
Sig.		.168	.060	.052	.094	.069	.764	.091

Appendix C2: Effect of different sizes of flasks on cell suspension culture of *Musa acuminata* cv. 'Berangan'

ANOVA

SGR

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.670	2	.335	7.395	.024
Within Groups	.272	6	.045		
Total	.942	8			

Homogeneous subset

SGR

Tukey HSD

Flask size	N	Subset for alpha = 0.05	
		1	2
1000 ml	3	4.5167	
500 ml	3	4.8900	4.8900
250 ml	3		5.1833
Sig.		.160	.284

Appendix C3: Effect of different photoperiods on cell suspension culture of *Musa acuminata* cv. 'Berangan'

Group Statistics

	Photoperiod	N	Mean	Std. Deviation	Std. Error Mean
SGR	18/6 hr	3	5.5100	.51069	.29484
	24/0 hr	3	5.8367	.35796	.20667

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
SGR	Equal variances assumed	.435	.546	-.907	4	.416	-.32667	.36006	-1.32636	.67302
	Equal variances not assumed			-.907	3.583	.421	-.32667	.36006	-1.37395	.72061

Appendix C4: Effect of different geometry of vessels (SF, BRT, pH-BRT) on cell suspension culture of *Musa acuminata* cv. 'Berangan'

ANOVA

SGR

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	4.496	2	3.748	160.619	.000
Within Groups	.140	6	.023		
Total	7.636	8			

Homogeneous subset

SGR

Tukey HSD

Geometry of vessel	N	Subset for alpha = 0.05		
		1	2	3
SF	3	5.6333		
BRT	3		6.6667	
pH-BRT	3			7.8667
Sig.		1.000	1.000	1.000

Appendix C5: Regeneration percentage between different culture vessels (SF, BRT, pH-BRT) on cell suspension culture of *Musa acuminata* cv. 'Berangan'

ANOVA

Regeneration percentage of banana plantlets

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	200.667	2	100.333	2.066	.208
Within Groups	291.333	6	48.556		
Total	492.000	8			

Homogeneous subset

Regeneration percentage of banana plantlets

Tukey HSD

Geometry of vessel	N	Subset for alpha = 0.05
		1
pH-BRT	3	62.3333
BRT	3	66.0000
SF	3	73.6667
Sig.		.195

Appendix C6: Number of regenerated shoots per 100 embryos

ANOVA

number of shoot per 100 embryos

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1828.667	2	914.333	1.873	.233
Within Groups	2929.333	6	488.222		
Total	4758.000	8			

number of shoot per 100 embryos

Tukey HSD

vessel	N	Subset for alpha = 0.05
		1
3	3	132.6667
2	3	140.3333
1	3	166.0000
Sig.		.234

Appendix C7: Number of regenerated plantlets per ml of SCV

ANOVA

number of plantlets per ml

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1658750.000	2	829375.000	.856	.471
Within Groups	5811250.000	6	968541.667		
Total	7470000.000	8			

number of plantlets per ml

Tukey HSD

vessel	N	Subset for alpha = 0.05
		1
3	3	7825.0000
2	3	8300.0000
1	3	8875.0000
Sig.		.442

Appendix C8: Percentage of normal plantlets

ANOVA

normal plantlets %

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	39.596	2	19.798	2.809	.138
Within Groups	42.293	6	7.049		
Total	81.889	8			

normal plantlets %

Tukey HSD

vessel	N	Subset for alpha = 0.05
		1
2	3	80.4429
1	3	83.3366
3	3	85.5663
Sig.		.121

Appendix C9: Protein content

ANOVA

% protein

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.011	2	.006	85.167	.000
Within Groups	.000	6	.000		
Total	.012	8			

% protein

Tukey HSD

Geometry of vessel	N	Subset for alpha = 0.05		
		1	2	3
3	3	.0767		
1	3		.1133	
2	3			.1633
Sig.		1.000	1.000	1.000

Appendix C10: Hydrogen peroxide content

ANOVA

umol H2O2/1 g FW

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.028	2	.014	22.674	.002
Within Groups	.004	6	.001		
Total	.032	8			

umol H2O2/1 g FW

Tukey HSD

Geometry of vessel	N	Subset for alpha = 0.05		
		1	2	3
1	3	.1658		
2	3		.2397	
3	3			.3022
Sig.		1.000	1.000	1.000

Appendix C11: CAT content

ANOVA

µmol H2O2/mg prot.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	89.910	2	44.955	9.851	.013
Within Groups	27.381	6	4.563		
Total	117.291	8			

µmol H2O2/mg prot.

Tukey HSD

Geometry of vessel	N	Subset for alpha = 0.05	
		1	2
1	3	3.1507	
2	3	4.2426	
3	3		10.3345
Sig.		.812	1.000

Appendix C12: SOD content

ANOVA

Units/100mgFW/min

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.438	2	.219	3.278	.109
Within Groups	.401	6	.067		
Total	.840	8			

Units/100mgFW/min

Tukey HSD

Geometry of vessel	N	Subset for alpha = 0.05
		1
2	3	7.0970
3	3	7.1652
1	3	7.5955
Sig.		.122

APPENDIX D: Conferences and publication

Appendix D1: Publication in Scientia Horticulturae

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Evaluation of a laboratory scale conventional shake flask and a bioreactor on cell growth and regeneration of banana cell suspension cultures

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ABSTRACT

It is important to develop a system to enhance the growth and regeneration of cells with limited totipotency into healthy plantlets. In this study, a semi automated cell suspension culture protocol for *Musa acuminata* cv. Berangan using bioreactor has been established. After eight months of culture, embryogenic calli generated from male inflorescences were transferred to liquid medium. Cell suspension cultures showed better cell growth when cultured in the M2 modified liquid medium containing 2% (w/v) sucrose with an initial inoculum density of 1:50 (cells:medium). The yield of cell suspension cultures was increased to 165% and 210%, respectively, when inoculated in 5 l balloon type bubble column bioreactors (BTBCBs) without pH control and with pH maintained at 5.7, over 14 days of culture. It was also shown that the growth yield was 3-fold higher when cultured in pH-controlled BTBCB, followed by 2.5-fold in uncontrolled-pH BTBCB and 2.2-fold in shake flask culture in comparison to initial culture (day 1). In all growth vessels tested, catalase (CAT) activity was found to be correlated with hydrogen peroxide (H_2O_2) concentration, suggesting its effective scavenging activity, whereas no significant difference was observed for superoxide dismutase (SOD). More than 60% of the embryos started to form shoots after 2 weeks of culture on M4 medium for all growth vessels tested. Potentially, this protocol may help in mass producing disease-free and high yielding banana as it provides a comparable cell growth rate producing normal regenerants in comparison to the conventional shake flask cultures.

1. Introduction

Banana belongs to the family of Musaceae. It is one of the important staple fruit crops in the tropical region and developing countries as its nutritional status is higher than other common tropical fruits (Sultan et al., 2011). Banana ranked fourth in terms of fruit crop worldwide with an annual production of approximately 100 million tons (Aquil et al., 2012). Currently, Cavendish sub group (AAA) is the most popular commercial cultivar of banana in the international trade (Robinson and Saúco, 2010). In Malaysia, there are about 27,500 ha under cultivation of approximately 50 banana varieties, including Berangan (*Musa* AAA), Rastali (*Musa* AAB), Abu (*Musa* ABB), Awak (*Musa* ABB) and Mas (*Musa* AA) (Jalil et al., 2003; Tee et al., 2011). Berangan (AAA) is widely cultivated due to its flavor and good storage qualities. Traditionally, banana is propagated using field-collected suckers or corms (Msogoya et al., 2011). However, this method is slow as only 5 to 10 suckers are produced per year (Makara et al., 2010) due to its long natural life cycle, sterility and polyploidy characteristics. Moreover, it is easy to transmit numerous diseases from old to new banana plantations along the generation route (Msogoya et al., 2011). Thus, there is a need to establish an efficient and less labor-intensive method for large-scale production of good quality cultivar Berangan in order to be competent as an export fruit crop.

Somatic embryogenesis has been adapted as an alternative method to mass produce disease-free planting materials. It has a potential to allow cryopreservation and to provide a valuable tool

Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; ANOVA, analysis of variance; BAP, 6-benzylaminopurine; BSA, bovine serum albumin; BTBCB, bubble type bubble column bioreactor; CAT, catalase; IAA, indoleacetic acid; MS, Murashige and Skoog; NAA, 1-naphthaleneacetic acid; PVPP, polyvinylpyrrolidone; PMSF, phenylmethylsulfonylfluoride; ROS, reactive oxygen species; SCV, settled cell volume; SOD, superoxide dismutase.

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for genetic improvement program (Bhojwani and Dantu, 2013). Low percentage of somaclonal variation is also an additional advantage of this technique. Panis et al. (1990) reported that only 0.7% of somaclonal variants were recorded for Bluggoe ABB cultivar using somatic embryogenesis compared to conventional micropropagation (0–70%) (Vuylsteke, 1989). Furthermore, this method has the advantages of scaled-up propagation using bioreactor (Shibli et al., 2012). Over the years, somatic embryogenesis has been reported for more than 500 plant species, including oil palm, citrus, corn and coffee (Bhojwani and Dantu, 2013). Numerous banana cultivars have been cultured via embryogenesis, such as Bluggoe (*Musa* ABB) (Dhed'a et al., 1991), Grande Nain (*Musa* AAA) (Becker et al., 2000), Rastali (*Musa* AAB) (Ganapathi et al., 2001) and Mas (*Musa* AAB) (Jalil et al., 2003) using different types of explants. Immature male inflorescences are the most responsive for producing embryogenic callus.

Traditionally, mass propagation of cell suspension culture is carried out in shake flasks. The parameters to support growth of cells are limited to media composition, temperature and light conditions. Using modern bioreactors, other parameters such as dissolved oxygen and pH can be controlled. A bioreactor is a method for propagating cells, tissues, somatic embryos and plantlets in liquid suspension (Paek et al., 2005; Lee et al., 2011). Optimum plant growth conditions can be conveniently achieved in a bioreactor by regulating various chemical and physical factors, including mixing, gaseous composition, efficient oxygen transfer, pH and hydrodynamic forces (Dong et al., 2013). To date, only a few studies have been reported on banana cell cultures using bioreactor. For instance, Kosky et al. (2002) propagated tetraploid banana hybrid (FHIA-18) using 2 l CMF-100 (CHEMAP AG, Switzerland) bioreactor and the plants produced were phenotypically normal. Recent study carried out by Dong et al. (2013) demonstrated a great potential of using bioreactor to produce suspension cells of *Panax ginseng*.

The critical cell culture conditions, which impact cell growth, plantlet productivity and quality, need to be optimized and controlled during bioreactor operation. Previous studies indicated that sucrose concentration, inoculum size and medium pH greatly affected cell growth performance (Ferri et al., 2011; Lee et al., 2011). Therefore, the present study was carried out to evaluate a laboratory scale conventional shake flask and a balloon type bubble column bioreactor (BTBCB) on cell growth and regeneration of banana cell suspension.

2. Materials and methods

2.1. Plant material

Inflorescence male buds of *M. acuminata* cv. Berangan (AAA) obtained from the local market at Petaling Jaya, Malaysia, were used as explants to initiate callus. The outer bracts of inflorescence buds were removed to 5–6 cm. Surface sterilization was carried out by submerging and shaking the explants in 70% (v/v) ethanol for 15 min inside a laminar air flow cabinet followed by three rinses with sterile distilled water. The cleaned explants were trimmed aseptically to 1–2 cm in length. A total of 15 immature male flower clusters occupying positions 1 to 15 (first being the flower cluster closest to the meristematic dome) were removed under microscope and inoculated on modified M1 solid medium (Jalil et al., 2003) containing MS basal salts (Murashige and Skoog, 1962) supplemented with Dhed'a vitamins (Dhed'a et al., 1991), 100 mg l^{-1} myo-inositol (Sigma, USA), 1 mg l^{-1} biotin, 1 mg l^{-1} indoleacetic acid (IAA) (Sigma, USA), 1 mg l^{-1} 1-naphthaleneacetic acid (NAA) (Sigma, USA), 4 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) (Sigma, USA), 10 mg l^{-1} ascorbic acid (Sigma, USA), 30 g l^{-1} sucrose (Sigma, USA), and 2 g l^{-1} gelrite (Sigma, USA). The medium was adjusted to pH 5.7 prior autoclaving at 121 °C for 20 min. All cultures were incubated at 25 ± 2 °C in dark condition and observed every two weeks until embryogenic callus was obtained.

2.2. Establishment of cell suspension culture

Translucent embryos and friable embryogenic callus were cultured into 50 ml conical flasks containing 10 ml M2 liquid medium (Côte et al., 1996) supplemented with MS basal salts, MS vitamins, 10 mg l^{-1} ascorbic acid, 1.1 mg l^{-1} 2,4-D, 250 μl l^{-1} *trans*-zeatin (Duchefa, Netherland), 30 g l^{-1} sucrose and 100 mg l^{-1} myo-inositol. The cultures were allowed to settle before transferred into 250 ml shake flask containing 50 ml M2 liquid medium after 30 days. Subsequently, the cell suspension cultures were subcultured by replacing 25 ml used medium with equal volume of fresh medium every 14 days. Cell suspension cultures were incubated at 25 ± 2 °C under continuous shaking condition of 90 rpm with a light intensity of 31.4 μmol m^{-2} s^{-1}.

2.3. Effect of sucrose and initial inoculum density in shake flask cultivation

To determine the effects of sucrose and initial inoculum density on cell growth, cell suspension cultures were inoculated in M2 liquid medium supplemented with different concentrations of sucrose (10, 15, 20, 25, 30 and 35 g l^{-1}) and different initial inoculum densities (0.5, 1.0, 1.5 and 2.0 ml) under a light intensity of 31.4 μmol m^{-2} s^{-1}. The growth of cell suspension cultures was measured by settled cell volume (SCV) after 14 days of culture and the pH of medium was measured at two-day intervals. Specific growth rate is defined as the increase in cell mass per unit time and was calculated using the following formula:

$$\text{Specific growth rate, } \mu_{\max}(day^{-1}) = \frac{\ln(SCV\ day\ 14) - \ln(SCV\ day\ 1)}{14}$$

2.4. Bioreactor cultivation

To study the growth of banana cells in scale-up environment, the cell suspension obtained from shake flask was cultured in a 5-liter balloon type bubble column bioreactor (BTBCB) fabricated by Fermetec Resources Sdn. Bhd., Malaysia (Fig. 1). The pH of medium was recorded every two days throughout 14 days of culture. A total of 20 ml SCV from shake flask was used as starting inoculum in 1 l culture medium. For another BTBCB cultures, the pH of medium was automatically controlled at 5.7 with 0.5 M NaOH or 0.5 M HCl using an automated controller fabricated by Fermetec Resources Sdn. Bhd. (Fig. 1). The specific growth rate of cells was calculated for each BTBCB every two days over 14 days. The SCV was determined by settling the cells in the bioreactor for 10 min and record the SCV from the scale bar on the BTBCB. The air flow rate was adjusted to 100 ml min^{-1} during cultivation to achieve a homogenous mixing stage. The cultivation temperature in bioreactors was controlled at 25 ± 2 °C under light intensity of 31.4 μmol m^{-2} s^{-1}.

2.5. Embryo development and regeneration

All suspension cells were sieved through 425 μm mesh size sieve (Endocotts Limited, England) before shoot regeneration. The cell density was adjusted to 20% SCV in a Falcon tube. 200 μl of the adjusted cell suspension cultures were transferred and cultured on development medium (M3) containing MS basal salts, MS vitamins, 30 g l^{-1} sucrose and 2 g l^{-1} gelrite. All cultures were maintained at 25 ± 2 °C under dark condition. A mix population of torpedo and heart-like shaped embryos were then transferred

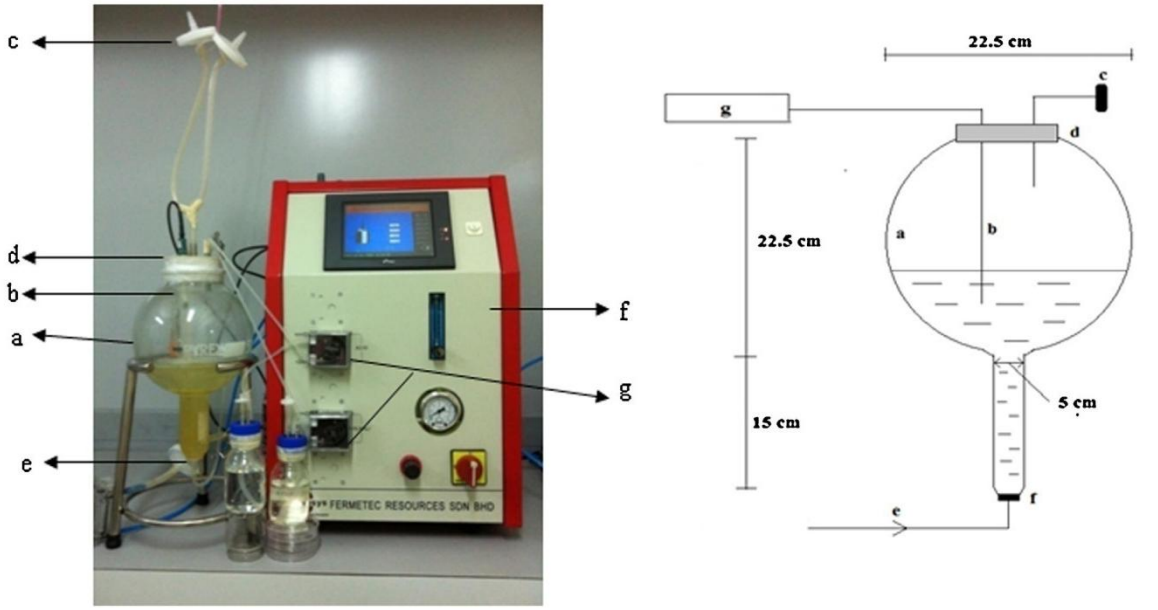

Fig. 1. Configuration of (a) 5 l balloon type bubble column bioreactor (BTBCB), (b) pH meter, (c) vent, (d) silicon cap, (e) filtered air, (f) sintered glass and (g) automated controller.

to modified regeneration medium (M4) (Jalil et al., 2003) consisting of MS basal salts, MS vitamins, 30 g l^{-1} sucrose, and 8 mg l^{-1} 6-benzylaminopurine (BAP) and cultured at 25 ± 2 °C under light condition. The percentage of shoot regeneration was determined by calculating the number of clumps developed into shoots over total number of torpedo and heart-like shaped embryos.

2.6. Protein extraction and estimation

1 g fresh weight of cells at day 14 of culture were frozen in liquid nitrogen and ground into a fine powder in 2.0 ml of 50 mM potassium phosphate buffer (pH 7.0) containing 2% (w/v) polyvinylpyrrolidone (PVPP) and 1 mM phenylmethylsulfonylfluoride (PMSF). The mixture was centrifuged at 10,000 rpm at 4 °C for 10 min. The supernatant was then transferred to a new tube and centrifuged at the same speed for another 10 min. The protein content was determined by Bradford method (1976) using bovine serum albumin (BSA) as a standard.

2.7. Determination of hydrogen peroxide (H_2O_2) and antioxidant enzyme activities

H_2O_2 content of the cells was measured according to Sergiev et al. (1997) with a H_2O_2 standard curve. Catalase (CAT) activity was measured according to the modified method by Baque et al. (2011). Superoxide dismutase (SOD) activity was measured according to Beauchamp and Fridovich (1971). The unit of SOD (SOD_{50}) is defined as the amount of SOD required to cause 50% inhibition of the oxidation.

2.8. Statistical analysis

All experiments were performed in three replicates. The data were recorded and analyzed using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test at a significance level of 5%.

3. Results and discussion

3.1. Initiation of somatic embryogenesis

Embryogenic calli of *M. acuminata* cv. Berangan (AAA) were initiated from the male inflorescence explants after 8 months of culture on modified M1 medium (Fig. 2). In the present study, 2,4-D (4 mg l^{-1}) was used to initiate cell de-differentiation to form

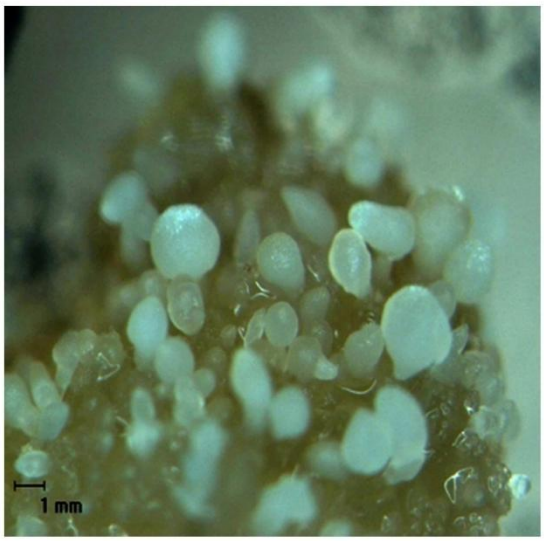

Fig. 2. Establishment of embryogenic cell suspension. Translucent embryos on friable callus of *Musa acuminata* cv. Berangan observed on M1 media after 8 months of culture.

embryogenic tissue following protocols established by Jalil et al. (2003). Induction of somatic embryogenesis is usually restricted to certain responsive cells of the explant under influence of auxins or other plant growth regulators (Rai et al., 2007). 2,4-D is considered to be one of the most effective auxins for the induction of somatic embryogenesis in plant tissue culture. Mujib and Šamaj (2006) reported that 2,4-D is able to enhance the cell division and simultaneously terminate the cell elongation. In our experience, it has been observed that the flower clusters became brown and swollen at the base after two weeks of culture, probably due to the presence of phenolic compounds and cell division activities. Exudation of a large quantity of phenolic compounds into medium might be toxic to the cells and caused cell death (Ko et al., 2009). Therefore, in this study, ascorbic acid was added as an antioxidant agent to reduce browning and to curtail the exudation of phenolic compounds by scavenging reactive oxygen species produced from the damaged explants (Titov et al., 2006).

The duration of embryogenic callus formation in *M. acuminata* cv. Berangan was longer (8 months) compared to other banana cultivars, such as Mas and Grande Nain (5–6 months) (Côte et al., 1996; Jalil et al., 2003). This may be due to the cultivar differences where banana cultivar AAA group was less responsive to somatic embryogenesis compared to AAB and ABB cultivar groups. Assani et al. (2002) also reported that the highest frequency of embryo formation was recorded in the AA genome group, followed by AAB and AAA genomes. Besides, the frequency of somatic embryo formation was also influenced by the type of explant. Our preliminary results indicated that flower clusters at positions 8 to 12 produced the highest percentage of somatic embryos (80%) (Fig. 3). No embryogenic callus was formed from flower clusters at positions 1 to 7. This coincides with the study carried out by Jalil et al. (2003), where more than 50% of flower clusters at positions 4 to 11 formed embryogenic callus. Of these, 18% of embryogenic callus was obtained from the flower cluster number 9. Wei et al. (2005) found that floral cluster from numbers 6 to 12 generated higher frequency of embryogenic callus compared to other positions.

3.2. Establishment of cell suspension culture

Whitish embryogenic callus and translucent somatic embryos (Fig. 2) were transferred into modified M2 medium to establish cell suspension culture consisting of heterogeneous globules. The embryogenic callus appear as heterogeneous suspension with clumps of embryos during the first few days of transfer. Homogeneous, yellowish and fine embryogenic cell suspension cultures (an accepted embryogenic characteristics of banana cell suspension; Ganapathi et al., 2001; Jalil et al., 2003) were obtained after two months of culture. Similar finding was reported in *Musa* Rasthali by Ganapathi et al. (2001).

The growth of banana cell suspension cultures recorded a lag phase for the first four days and gradually increased to the highest specific growth rate at 5×10^{-3} day^{-1} after two weeks of culture. The cells started to turn brown and eventually not growing after 16 days of culture. The pH of media in the shake flasks decreased drastically within the first five days from 5.7 to 3.8, whereas the cell growth was increased two-fold from day 7 to 14. The sudden drop of the pH might be due to the uptake of NH_4^+ and NO_3^- by plant cells in the culture medium (Chung et al., 2006). Therefore,

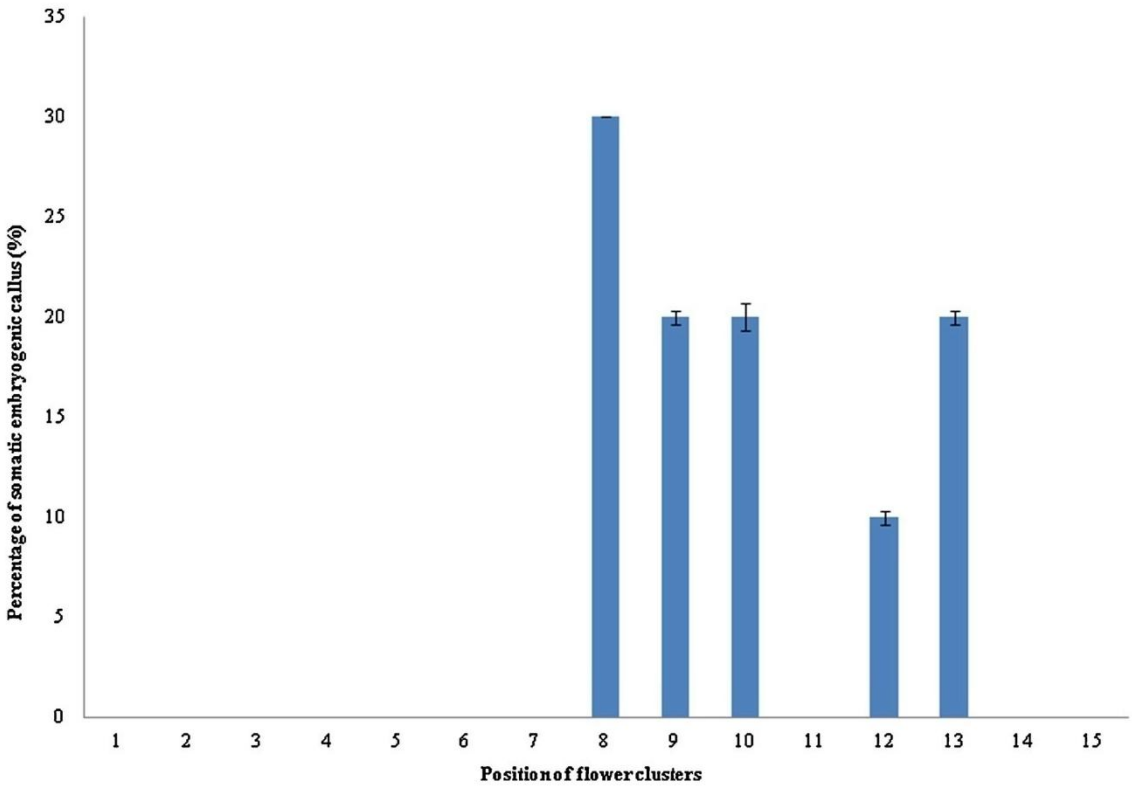

Fig. 3. The frequency of forming somatic embryo at different flower cluster positions of *Musa acuminata* cv. Berangan.

under similar conditions, the maximum growth of cell suspension cultures was expected on day 14 of culture.

3.3. Effects of sucrose and initial inoculum densities on cell growth

Cell suspension cultures demonstrated different responses according to the concentrations of sucrose in the culture media. The medium containing 20 g l^{-1} sucrose produced the highest SCV of yellowish homogeneous cell suspensions. On the other hand, lower SCV and less vigorously growing brownish cells were observed when cultured in the medium containing sucrose less than 20 g l^{-1} or more than 25 g l^{-1} (Fig. 3). High level of sucrose might retard the cell growth and increased osmotic stress that could lead to cell death (Al-Khayri and Al-Bahrany, 2002; Baque et al., 2011). Sucrose is the most common carbohydrate found in phloem sap. It has been extensively used in plant cell cultures to provide energy and to maintain osmotic pressure in the cells (Wu and Ho, 1999). It also acts as signalling molecules for plant growth and development, stress resistance and metabolism (Moghaddam and Van den Ende, 2012). Several studies demonstrated that sucrose concentration is important for cell growth, root culture and production of secondary metabolites (Cui et al., 2000, 2010a, 2010b; Ferri et al., 2011).

The starting inoculum density of cell suspension culture is also another essential parameter as it can influence the cell growth, such as cell biomass (Gorret et al., 2004), metabolite accumulation (Zhong and Yoshida, 1995) and hairy root growth (Yu et al., 1996; Dehghan et al., 2012). Our results indicated that 1.0 ml SCV in 50 ml culture medium exhibited the highest specific growth rate (5.3×10^{-2} day^{-1}) with vigorously growing cells (Fig. 3). In contrast, relatively higher amount of brown cells were observed when 0.5 ml SCV was used. This might be due to the low density of cells resulting in longer lag phase or programmed cell death by the plant itself (McCabe and Leaver, 2000). Higher initial SCV (1.5 and 2.0 ml) produced less number of vigorously growing cells compared to 1.0 ml SCV.

3.4. Scale-up cultivation of banana cells in BTBCB

A BTBCB with 1 l working volume was inoculated with 20.0 ml SCV cell suspension culture (1:50). The pH of medium in the BTBCB decreased to 3.8 at day 5 of culture with a rate of Δ0.38 pH unit day^{-1}. Similar results were recorded in the shake flask cultures. For the growth rate, the culture in the BTBCB was significantly ($p < 0.05$) higher (6.5×10^{-2} day^{-1}) than shake flask cultures (5.3×10^{-2} day^{-1}) (Fig. 4). Although column bioreactor is suitable for plant micropropagation, however, clogging in the vent of bioreactor often occurs due to excessive foaming (Paek et al., 2001). Hence, a modified bioreactor (BTBCB) designed by Paek et al. (2005) was used in this study as it could reduce the foaming by introducing a concentric tube at the base of the bioreactor and wider surface area at the top of bioreactor (Fig. 1). Large scale plant production using BTBCB has been reported in *Panax ginseng*, orchids and other valuable plants (Paek et al., 2005).

In another experiment, banana cell suspension cultures were maintained in a BTBCB with pH control. The cell cultures with pH-controlled at 5.7 showed higher cell growth rate (7.9×10^{-2} day^{-1}) compared to cells grown in BTBCB system with uncontrolled-pH (6.5×10^{-2} day^{-1}) and shake flasks (5.3×10^{-2} day^{-1}) (Fig. 4). The highest growth yield of cultures (SCV day 14/SCV day 1) was recorded in pH-controlled BTBCB (3.01), followed by uncontrolled-pH BTBCB (2.53) and shake flask (2.23). The growth profile and growth rate of banana cell suspension cultures in shake flask, uncontrolled-pH BTBCB and pH-controlled BTBCB are shown in Fig. 5. The pH of the culture medium was fixed at 5.7, as most of the salts would be available in soluble form at this pH allowing ease of absorption and thus promoted fast growth and differentiation (Murashige and Skoog, 1962). This underlined the importance of pH control in cell suspension cultures as the nutrients uptake is greatly affected by the pH milieu (George et al., 2008). Chung et al. (2006) also indicated that a strong acidic condition may also affect the proliferation of cell clusters.

3.5. Embryo development and regeneration

The cell suspension cultures were first developed on M3 medium in dark condition to induce adventitious buds and transferred to M4 medium to allow chloroplast formation and shoots development (Fig. 6). More than 60% of the embryos started to form shoots after 2 weeks of culture on M4 medium (Table 1). The highest frequency of regeneration (74%) was obtained from the shake flask, whereas the lowest frequency (62%) was recorded from the pH-controlled bioreactor. However, differences between the growth vessels were not significant. Of all regenerants, the highest

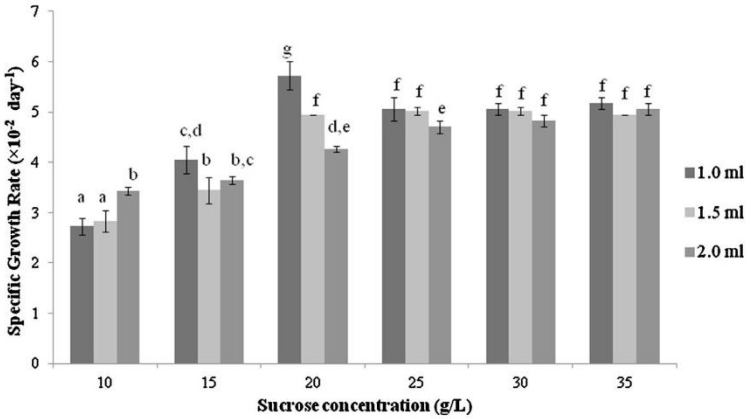

Fig. 4. Effects of sucrose and initial inoculum density on *Musa acuminata* cv. Berangan cell suspension growth. Results represent mean ± standard deviation of three replicated experiments after 14 days of culture. Different letters indicate a significant difference at $p < 0.05$.

Table 1
Embryo development and regeneration of *Musa acuminata* cv. Berangan cell suspension cultures.

Growth vessel	Developed embryos (%)	Number of shoots developed per 100 developed embryo	Number of plantlets generated per ml (SCV)	Normal plantlets (%)
SF	74 ± 5[a]	166 ± 15[a]	8800 ± 30[a]	80 ± 2[a]
BRT	66 ± 3[a]	140 ± 11[a]	8300 ± 30[a]	80 ± 2[a]
pH-BRT	62 ± 4[a]	134 ± 13[a]	7825 ± 11[a]	87 ± 2[a]

The results represent the mean ± standard error ($n = 3$). Different letters indicate values are significantly different at $p < 0.05$.

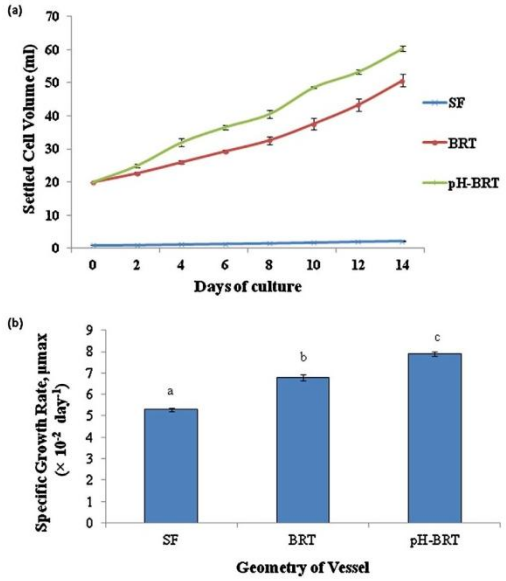

Fig. 5. (a) Growth profile and (b) average specific growth rate of *Musa acuminata* cv. Berangan in shake flask (SF), no pH control in BTBCB (BRT) and pH-controlled BTBCB (pH-BRT) over 14 days of culture. Results represent mean ± standard deviation of three replicated experiments after 14 days of culture. Different letters indicate a significant difference at $p < 0.05$.

percentage of normal plantlets (87%) with respect to morphological characteristic compared to donor plant was recorded from the pH-controlled bioreactor. The characteristics of abnormal plantlets were with references to Côte et al. (2000). Dwarf, giant, mosaic-like, deformed leaves and fasciated-leafed plantlets were determined as abnormal.

3.6. Determination of H_2O_2 and antioxidant enzyme activities

Plants counteract the toxicity of reactive oxygen species (ROS) generated from both stressed and unstressed cells by activating enzymatic (SOD, CAT, POD and APX) or non-enzymatic defense system. In this study, the endogenous H_2O_2 content and antioxidative enzyme activities of the cells grown in shake flask and bioreactors were investigated (Table 2). It was found that endogenous H_2O_2 concentration were higher in cells in pH-controlled BTBCB (0.30 μmol g^{-1} FW) compared to uncontrolled-pH BTBCB (0.24 μmol g^{-1} FW) and shake flask (0.17 μmol g^{-1} FW). H_2O_2 plays a very important role in response to stress and physiological activities in plants and acts as a signaling molecule when plants are under severe environmental conditions. The cellular content of H_2O_2 has been reported to reflect damage from oxidative stress (Meratan et al., 2009; Abbasi et al., 2011; Ozden and Karaaslan, 2011). Quan et al. (2008) have shown that high levels of H_2O_2 can promote programmed cell death but that at low levels the compound can promote plant growth and development.

Our results also indicated that SOD activity for all types of growth vessels tested was not significant ($p < 0.05$). SOD operates as the first line of defense against ROS by catalyzing the dismutation of O_2^- into H_2O_2 and O_2 that can be scavenged by CAT and glutathione reductase (GR) in the Halliwell–Asada pathway (Chen et al., 2013). This study indicated the SOD activity did not correlate with the level of H_2O_2 in our studies. This might be due the involvement of different mechanisms in dealing with oxidative stresses. For instance, Moussa and Abdel-Aziz (2008) reported that SOD activity was significantly increased in wheat under drought condition but not water stress. On the other hand, CAT activity was found to be correlated with H_2O_2 concentration, in which a higher level of CAT activity was detected from the cells grown in pH-controlled BTBCB. High activity of CAT suggests its effective scavenging activity of

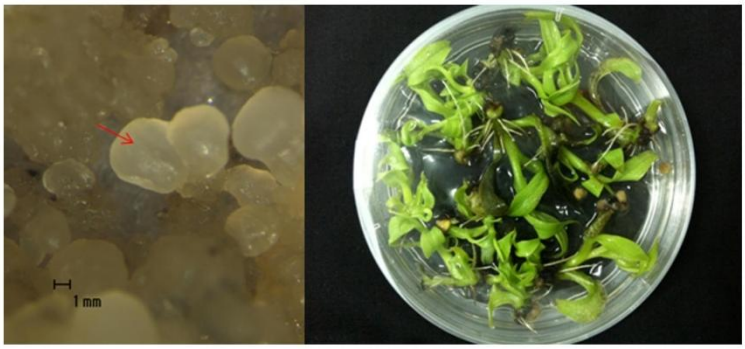

Fig. 6. (a) Mature embryos on M3 medium after one month of culture and (b) regenerated banana plantlets of *Musa acuminata* cv. Berangan.

Table 2
The endogenous H_2O_2 content and antioxidative enzyme activities of *Musa acuminata* cv. Berangan cells grown in shake flask and bioreactors.

Growth vessel	H_2O_2 (μmol g^{-1} FW)	CAT (μmol H_2O_2 mg^{-1} protein)	SOD (Unit 100 mg^{-1} FW min^{-1})
SF	0.17 ± 0.01^a	3.15 ± 0.63^a	7.60 ± 0.04^a
BRT	0.24 ± 0.02^b	4.24 ± 0.71^a	7.10 ± 0.18^a
pH-BRT	0.30 ± 0.01^c	10.33 ± 1.91^b	7.17 ± 0.18^a

The results represent the mean ± standard error ($n = 3$). Different letters indicate values are significantly different at $p < 0.05$.

H_2O_2 produced in cells. Although the formation of ROS might affect proliferation capacity of plant cells, data presented here showed that cells grown in pH-controlled BTBCB were able to produce significant higher growth rate than uncontrolled-pH growth vessels and shake flasks in normal condition.

4. Conclusion

In conclusion, the study showed that in a pH-controlled BTBCB, there is a good potential to cultivate banana cell suspension cultures at relatively dense level and significant growth rate as compared to the traditional shake flasks method. High frequency of normal plantlets obtained from banana cell suspension grown in pH-controlled BTBCB indicated a great potential of using this bioreactor system for automated large scale banana plantlets production.

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Appendix D2: Abstract and poster for 17th Biological Sciences Graduate Congress (BSGC), Bangkok, 2012

OPTIMIZATION OF *MUSA ACUMINATA* CV. BERANGAN EMBRYOGENIC CELL SUSPENSION IN AIR-DRIVEN BALLOON TYPE BIOREACTOR

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Musa acuminata cv. Berangan is one of the main commercially grown banana cultivar in Malaysia. It is favourable in the domestic market because of its high yield, flavour and good storage qualities. The planting materials of Berangan which are commercial grown are mostly dependant on *in vitro* source produced using tissue culture technology. This is mainly to ensure consistent elite quality and uniform fruiting. Application of automated bioreactor provides better strategy to reduce labour cost and time while promoting maximum growth of cells suspension. Comparatively, conventional mass propagation in shake flasks requires more space and power consumption. The aim of this research is to develop an optimized bioreactor system for mass propagation of banana embryogenic cell suspension culture. Embryogenic calli were produced from male inflorescence of Pisang Berangan. Liquid suspension composed of embryogenic cells was established for steady and continuous supply of clonal materials. The effect of sucrose concentration and inoculation volume on the cell growth for bioreactor was pre-optimized in shake flasks. The parameters of pH and air flow affecting propagation of cells were further optimized in an automated air-driven balloon type bioreactor.

Optimisation of *Musa acuminata* cv. Berangan Embryogenic Cell Suspension in Air-driven Balloon Type Bioreactor

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[1] Centre for Research in Biotechnology for Agriculture (CEBAR), Institute of Biological Sciences, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia.
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INTRODUCTION

❑*Musa acuminata* cv. Berangan is one of the main commercially grown banana cultivar in Malaysia. It is favourable in the domestic market because of its high yield, flavour and good storage qualities.

❑The planting materials of Berangan are mostly dependant on *in vitro* source to ensure consistent elite quality and uniform fruiting.

❑Conventional mass propagation in shake flasks requires more space and power consumption.

❑Application of automated bioreactor provides better strategy to reduce labour cost and time while promoting maximum growth of cells suspension hence maximise number of planting materials.

OBJECTIVE

To develop an optimised bioreactor system for mass propagation of banana embryogenic cell suspension culture

METHODOLOGY

Explant sterilization and callus induction

↓

Establishment of embryogenic cell suspension culture

↓

Optimisation of embryogenic cell suspension culture in shake flask

↓

Optimisation of embroygenic cell suspension culture in bioreactor

↓

Embryo development and regeneration

ACKNOWLEDGEMENT
This project is supported and funded by UM-UMRG grant (RG013-09BIO) and MOSTI-SKIM SCIENCEFUND ER-BIOTEK (10-01-03-PE004).

RESULTS & DISCUSSION

❑Embryogenic callus was first observed after 8 months of culture in M1 media. Fig. 1a shows the embryogenic callus observed under microscope.

❑Fine embryogenic cell suspension was obtained after 2-3 months of culture (Fig. 1b).

❑The effect of sucrose concentration and inoculum density on cell suspension growth in shake flask is shown in Graph 1. 1 ml inoculum density and 20 g/L sucrose was the best condition for banana cell suspension cultures. Sucrose at concentration higher than 30g/L caused browning. This may be due to severe osmotic stress on the cells.

❑The effect of controlled pH on cell suspension growth in 5 L bioreactor is shown in Graph 2. Bioreactor culture with controlled pH system showed increased cell growth compared to bioreactor culture system without pH control. This may be due to maximum nutrient uptake throughout the culture period.

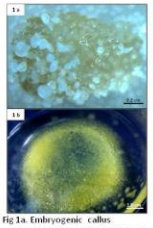
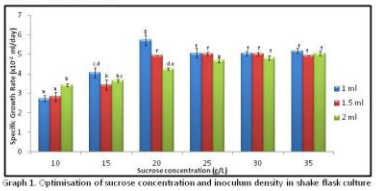
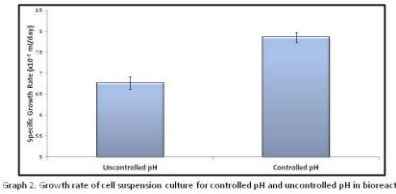


Fig 1a. Embryogenic callus
b. Embryogenic cell suspension

Fig 2. Automated bioreactor apparatus for cell culture



Graph 1. Optimisation of sucrose concentration and inoculum density in shake flask culture



Graph 2. Growth rate of cell suspension culture for controlled pH and uncontrolled pH in bioreactor

CONCLUSION

Mass propagation of banana embryogenic cell suspension culture in balloon bioreactor promoted cell growth at optimised parameters.

Appendix D3: Abstract and poster for International Conference on Crop Improvement (ICCI), Putra University Malaysia (UPM), 2013

POTENTIAL OF BALLOON TYPE BUBBLE COLUMN BIOREACTOR IN IMPROVEMENT OF BANANA (*MUSA ACUMINATA* CV. BERANGAN)

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Banana is one of the most important staple fruit crop and contributes significantly to food safety and security, especially in the tropical region. However, disease and pest infection has caused devastating loss worldwide. Therefore, it is important to establish an efficient and less labourious method to mass produce high quality planting materials in order to feed a growing population. In this study, embryogenic calli generated from male inflorescences of *Musa acuminata* cv. Berangan were used to establish cell suspension cultures in modified M2 liquid medium containing 2% sucrose. The established cell suspension cultures were subsequently transferred to a shake flask, uncontrolled-pH balloon type bubble column bioreactor (BTBCB) or pH-controlled BTBCB equipped with an automated controller. The yield of cell suspension cultures was increased to 165% and 210% over 14 days of culture when inoculated in BTBCBs without pH control and with pH maintained at 5.7, respectively. Our antioxidant enzyme activities assay revealed that catalase (CAT) activity was correlated with hydrogen peroxide (H_2O_2) concentration, suggesting its effective scavenging activity. Higher CAT activity (10.33 µmol H_2O_2/mg protein) was recorded in the pH-controlled BTBCB compared to other growth vessels tested, whereas no significant difference was found for superoxide dismutase activity. More than 86% pH-controlled BTBCB-derived plantlets were found to be normal with respect to morphological characteristics compared to donor plant, whereas only 80% of normal plantlets were recorded for the conventional shake flask. Our protocol demonstrates the feasibility of BTBCB to mass produce uniform and disease-free embryogenic cell suspension-derived bananas.

Potential of balloon type bubble column bioreactor in improvement of banana (*Musa acuminata* cv. Berangan)

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Abstract

Banana is one of the most important staple fruit crop and contributes significantly to food safety and security, especially in the tropical region. However, disease and pest infection has caused devastating loss worldwide. Therefore, it is important to establish an efficient and less labourious method to mass produce high quality planting materials in order to feed a growing population. In this study, embryogenic calli generated from male inflorescences of *Musa acuminata* cv. Berangan were used to establish cell suspension cultures in modified M2 liquid medium containing 2% sucrose. The established cell suspension cultures were subsequently transferred to a shake flask, uncontrolled-pH balloon type bubble column bioreactor (BTBCB) or pH-controlled BTBCB equipped with an automated controller. The yield of cell suspension cultures was increased to 165% and 210% over 14 days of culture when inoculated in BTBCBs without pH control and with pH maintained at 5.7, respectively. Our antioxidant enzyme activities assay revealed that catalase (CAT) activity was correlated with hydrogen peroxide (H_2O_2) concentration, suggesting its effective scavenging activity. Higher CAT activity (10.33 µmol H_2O_2/mg protein) was recorded in the pH-controlled BTBCB compared to other growth vessels tested, whereas no significant difference was found for superoxide dismutase activity. More than 86% pH-controlled BTBCB-derived plantlets were found to be normal with respect to morphological characteristics compared to donor plant, whereas only 80% of normal plantlets were recorded for the conventional shake flask. Our protocol demonstrates the feasibility of BTBCB to mass produce uniform and disease-free embryogenic cell suspension-derived bananas.

Objective

To evaluate a laboratory scale conventional shake flask and a balloon type bubble column bioreactor on cell growth and regeneration of banana cell suspension

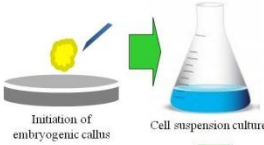
Methodology

Plant material

(**A**) Inflorescence male bud of *Musa acuminata* cv. Berangan and (**B**) The outer bracts of inflorescence buds were removed to 5-6 cm (Bar = 1 cm)

Surface disinfection

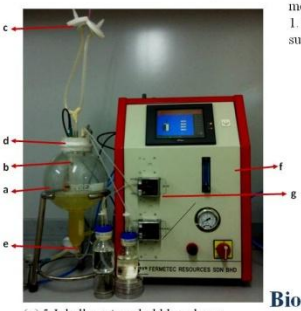
Surface disinfection was carried out by submerging and shaking the explants in 70% ethanol for 15 min followed by three rinses with sterile distilled water. The cleaned explants were trimmed aseptically to 1-2 cm in length



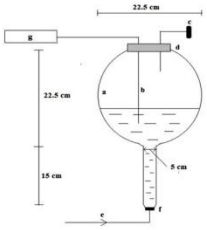
Establishment of embryogenic callus and cell suspension cultures

Callus was initiated on Murashige and Skoog (MS) basal salts supplemented with Dhed'a vitamins (Dhed'a *et al.*, 1991), 1 mg/l indoleacetic acid, 1 mg/l 1-naphthaleneacetic acid, 4 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), 30 g/l sucrose, and 2 g/l Gelrite

Embryogenic callus was cultured in liquid MS medium (Côte *et al.*, 1996) containing MS vitamins, 1.1 mg/l 2,4-D, 250 mg/l trans-zeatin, and 30 g/l sucrose



(**a**) 5-L balloon type bubble column bioreactor, (**b**) pH meter, (**c**) vent, (**d**) silicon cap, (**e**) filtered air, (**f**) sintered glass, and (**g**) automated controller



Bioreactor cultivation

A total of 20 ml SCV from shake flask was used as starting inoculum in 1 l culture medium in a 5 l balloon type bubble column bioreactor (BTBCB) fabricated by Fermetec Resources Sdn. Bhd., Malaysia

Results

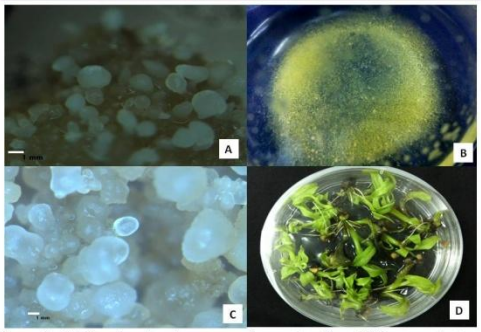


Figure 1: (**A**) Translucent embryos on embryogenic callus of *Musa acuminata* cv. Berangan observed on M1 media after 8 months of culture, (**B**) homogeneous cell suspension culture in M2 media (**C**) Translucent spheres and slightly torpedo shaped embryos on M3 medium after one month of culture, and (**D**) regenerated plantlets

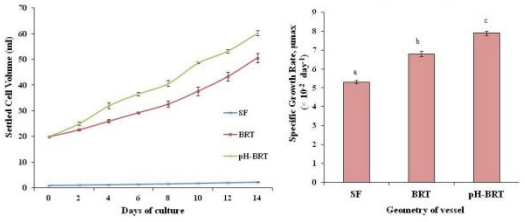


Table 1: The endogenous H_2O_2 content and antioxidative enzyme activities of the cells grown in shake flask and bioreactors

Growth vessel	H_2O_2 (µmol/g FW)	CAT (µmol H_2O_2/mg protein)	SOD (Unit/100 mg FW/min)
SF	0.17 ± 0.01[a]	3.15 ± 0.63[a]	7.60 ± 0.04[a]
BRT	0.24 ± 0.02[b]	4.24 ± 0.71[a]	7.10 ± 0.18[a]
pH-BRT	0.30 ± 0.01[c]	10.33 ± 1.91[b]	7.17 ± 0.18[a]

Results represent mean ± standard deviation of three replicated experiments after 14 days of culture
Different letters indicate a significant difference at $p < 0.05$

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Acknowledgements

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