ENHANCEMENT AND ISOLATION OF ZERUMBONE IN *Zingiber zerumbet* (L.) Smith CELL SUSPENSION AND ADVENTITIOUS ROOT CULTURES

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ENHANCEMENT AND ISOLATION OF ZERUMBONE IN Zingiber zerumbet (L.) Smith CELL SUSPENSION AND ADVENTITIOUS ROOT CULTURES

ABSTRACT

Zingiber zerumbet Smith is a highly valuable medicinal ginger belonging to Zingiberaceae family containing bioactive phytomedicinal compound called zerumbone (ZER). Limitation in Zingiberaceae conventional breeding techniques makes alternative approach through biotechnological plant tissue culture as the most practical route to obtain targeted bioactive compound(s). In this study, production of ZER was carried out using two *in vitro* culture systems namely cell suspension and adventitious roots. The establishment of cell suspension culture under selected set of physiochemical parameters was investigated for biomass growth and ZER production. It was found that the type of carbohydrate substrate, light regime, agitation speed, and incubation temperature showed significant effects on the production of ZER. The highest specific growth rate of cells was recorded in liquid Murashige and Skoog (MS) basal medium containing 3% sucrose with pH 5.7 and incubated under continuous shaking of 70 rpm with 16 hr photoperiod at 24°C. However, the yield of ZER in cell suspension cultures of *Z. zerumbet* was low and extracellular, thus making the compound enhancement and recovery complicated. Consequently, development and optimization of *Z. zerumbet* adventitious root (AdR) culture was carried out. From the results, the frequency of root response, number of roots per explant, root length, and ZER production were affected by the concentration and types of auxins used for both AdR initiated through direct and indirect organogenesis (AdRD and AdRId respectively). For roots multiplication, the highest specific growth rate was achieved in AdRId with an initial inoculum of 0.5 g fresh weight (FW) in full strength MS medium. Nevertheless, the amount of ZER was found to be highest in half strength MS in AdRD at 2520 µg g^{-1} DW (p<0.05). In order to enhance ZER production, elicitation strategy was employed on selected root cultures.

It was observed that salicylic acid (SA) was more efficient at lower concentration compared to methyl jasmonate (MeJA) in eliciting ZER secretion by the root culture. In contrast, the biomass harvested from SA-treated root culture was significantly lower to MeJA-treated culture, hence demanding longer period of cultivation prior to harvesting. Therefore, further optimization was carried out based on the time interval for supplementation of optimized MeJA concentration (800 µM) at 15, 20 and 25 days of cultivation. MeJA treatment during exponential phase (supplementation at day 15) significantly increased ZER expression at 42,777 $\mu g g^{-1}$ DW ($p<0.05$). The yield obtained was comparable to the ZER extracted from field-grown rhizome. Elicitor treatment to plant cell cultures whilst enhanced the production of secondary metabolite, it also induced oxidative stress, indicated by the increase in intracellular concentration of hydrogen peroxide (H_2O_2). Thus, the relationship between ZER production and the profiles of hydrogen peroxide (H_2O_2) concentration, catalase (CAT) and ascorbate peroxidase (APX) activities were investigated for untreated (control) and MeJA elicited roots. However, conclusive pattern relating the variables studied in the untreated control culture was absent. In elicited roots, the concentration of H_2O_2 was found to be significantly high, which was attributed to the oxidative stress response from elicitor addition. Since large-scale production is necessary for compound harvesting, the AdR of *Z. zerumbet* was propagated in a controlled balloon-type column bioreactor (BTBCB). From the results, volume of initial inoculum and aeration rate affected the specific growth rate and ZER production while pH affected the root biomass growth but showed no significant effect on ZER accumulation. Lower initial inoculum density (10 $g L^{-1}$) yielded the highest specific growth rate while ZER production was highest when initial inoculum density was 20 $g L^{-1}$. Optimum aeration rate at 1.0 $L min^{-1}$ resulted in a three-fold increment of initial biomass and highest ZER accumulation. In conclusion, AdR system with elicitation strategies showed promising results in ZER production

from *Z. zerumbet* root cultures, and this could be applied as a model system for large-scale production of similar plant metabolites.

Keywords: Lempoyang, elicitation, methyl jasmonate, salicylic acid, bioreactor

PENINGKATAN DAN PEMENCILAN ZERUMBON DALAM SEL AMPAIAN DAN AKAR ADVENTITIUS *Zingiber zerumbet* (L.) Smith

ABSTRAK

Zingiber zerumbet Smith adalah halia bernilai perubatan yang sangat berharga yang tergolong dalam famili Zingiberaceae yang mengandungi sebatian bioaktif fitoperubatan dikenali sebagai, zerumbon (ZER). Pengehadan dalam pembiakan konvensional Zingiberaceae membuatkan pendekatan bioteknologi melalui kultur tisu tumbuhan sebagai cara alternatif untuk mengeksploitasi sebatian bioaktif yang disasarkan. Dalam kajian ini, pengekstrakan ZER telah dijalankan dari dua sistem kultur *in vitro* iaitu sel ampaian dan akar adventitius. Penubuhan kultur sel ampaian dilakukan bagi set parameter fisiokimia yang terpilih untuk mengkaji pertumbuhan biojisim dan pengeluaran ZER. Keputusan menunjukkan bahawa jenis substrat karbohidrat, rejim cahaya, kelajuan agitasi, dan suhu pengeraman mempengaruhi penghasilan ZER. Kadar pertumbuhan spesifik tertinggi sel dicatatkan dalam medium cecair asas Murashige dan Skoog yang mengandungi 3% sukrosa dengan pH 5.7 dan dieram di bawah keadaan agitasi berterusan pada 70 rpm dengan pengkalaan cahaya 16 jam pada 24°C. Namun begitu, penghasilan ZER dalam sel ampaian *Z. zerumbet* adalah rendah dan berada extrasellular, oleh itu menjadikan strategi peningkatan kompaun adalah sukar. Dengan itu, pembangunan dan pengoptimuman kultur akar adventitius (AdR) bagi *Z. zerumbet* dilakukan. Dari keputusan, didapati bahawa kekerapan respons akar, bilangan akar per eksplan, panjang akar, dan penghasilan ZER dipengaruhi oleh kepekatan dan jenis auksin yang digunakan untuk kedua-dua AdR yang terhasil melalui organogenesis secara langsung dan tidak langsung (AdRD dan AdRId, masing-masing). Untuk penggandaan akar, kadar pertumbuhan spesifik yang paling tinggi dicapai dalam AdRId dengan inokulum awal sebanyak 0.5 g FW dengan kekuatan penuh MS manakala jumlah ZER adalah paling tinggi pada separuh kekuatan MS [2520 µg/g DW ($p<0.05$)]

dalam AdRD. Bagi meningkatkan penghasilan ZER, strategi elisitasi telah dilakukan ke atas kultur akar yang terpilih. Diperhatikan bahawa elisitasi menggunakan asid salisilik (SA) adalah lebih cekap pada kepekatan yang lebih rendah berbanding dengan metil jasmonat (MeJA) bagi elisitasi pengekstrakan ZER dalam kultur akar . Sebaliknya, biojisim yang dituai untuk akar terawat SA adalah jauh lebih rendah secara signifikannya terhadap akar terawat MeJA, dengan ini ianya memerlukan tempoh kultivasi yang lebih lama sebelum penuaian. Oleh itu, pengoptimuman lanjut telah dijalankan berdasarkan selang masa suplementasi bagi kepekatan MeJA yang telah teroptimum (800 µM) pada 15, 20 dan 25 hari kultivasi. Rawatan MeJA semasa fasa eksponen (suplementasi pada hari 15) mendapati terdapat peningkatan yang ketara dalam pengekspresan ZER pada 42.777 µg/g DW (p <0.05). Hasil yang didapati adalah setanding dengan ZER yang diekstrak daripada rizom yang ditanam di lapangan. Rawatan elisitor ke atas kultur sel tumbuhan bukan sahaja meningkatkan penghasilan metabolit sekunder tetapi juga mengaruhkan tekanan oksidatif, yang ditunjukkan dengan kehadiran hidrogen peroksida (H_2O_2). Oleh itu, hubungan antara penghasilan ZER dan profil aktiviti hidrogen peroksida (H_2O_2), katalase (CAT) dan (askorbat peroksidase) APX dikaji untuk akar tidak terawat (kawalan) dan akar yang dielisitasi dengan MeJA. Walaubagaimana pun, diperhatikan bahawa tiada corak yang ketara berkaitan hubungan antara pembolehubah yang dikaji dalam kultur kawalan yang tidak terawat. Bagiakar yang terelisitasi, kepekatan H_2O_2 didapati agak tinggi dan ini boleh dikaitkan dengan tindak balas tekanan oksidatif berikut penambahan elisitor tersebut. Memandangkan penghasilan berskala besar adalah perlu bagi penuaian kompaun, oleh itu AdR bagi *Z. zerumbet* telah dipropagasi dalam keadaan yang terkawal dalam bioreaktor jenis turus belon (BTBCB). Berdasarkan keputusan yang diperolehi, jumlah inokulum awal dan kadar aliran udara mempengaruhi kadar pertumbuhan spesifik dan penghasilan ZER manakala pH hanya mempengaruhi pertumbuhan biojisim akar tetapi

tidak menunjukkan kesan yang besar ke atas pengumpulan ZER. Kepadatan inokulum awal yang lebih rendah (10 gL^{-1}) memberikan kadar pertumbuhan spesifik yang paling tinggi manakala penghasilan ZER adalah paling tinggi bagi inokulum yang lebih tinggi (20 gL^{-1}). Aliran udara yang optimum pada 1.0 $Lmin^{-1}$ menunjukkan tiga-kali ganda peningkatan biojisim dari biojisim awal semasa inokulasi dan memaparkan pengumpulan ZER tertinggi. Kesimpulannya, sistem AdR dengan strategi elisitasi menunjukkan hasil yang boleh diharapkan dalam penuaian ZER dari kultur akar *Z. Zerumbet* dan ianya boleh digunakan sebagai sistem model untuk penghasilan secara besar-besaran kompaun yang bernilai komersial dalam penyelidikan di masa hadapan.

Katakunci: Lempoyang, elisitasi, metil jasmonat, asid salisilik, bioreaktor

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α	:	alpha
β	:	beta
2,4-D	:	2,4-dichlorophenoxyacetic acid
λ	:	lambda
<	:	less than
µg	:	microgram
µmol	:	micromole
µg/g	:	microgram per gram
µm	:	micrometre
µl/L	:	microliter per litre
µM	:	micromolar
>	:	more than
±	:	more less
%	:	percentage
ACN	:	acetonitrile
AdR	:	adventitious root
AdRId	:	adventitious root through indirect organogenesis
AdRD	:	adventitious root through direct organogenesis
APX	:	ascorbate peroxidase
AR (grade)	:	analytical (grade)
BA	:	6-benzyladenine
BAP	:	benzylaminopurine
BTBCB	:	balloon-type bubble column bioreactor
C	:	carbon
CAT	:	catalase
CHO	:	Chinese hamster ovary cell

cm	:	centimetre
d	:	day
DB	:	dried biomass
DCM	:	dichloromethane
DNA	:	deoxyribonucleic acid
DW	:	dry weight
e.g	:	for example
et al.	:	Latin : et alii or English : and others
EDTA	:	ethylenediaminetetra acetic acid
FAA	:	formaldehyde-absolute ethanol acetic acid
FW	:	fresh weight
g	:	gram
g/L	:	gram per litre
GC	:	gas chromatography
GC/MS	:	gas chromatography mass spectrometry
h	:	hour
H_2O_2	:	hydrogen peroxide
HCl	:	hydrochloric acid
HPLC	:	high performance liquid chromatography
IAA	:	indole-3-acetic acid
IBA	:	indole-3-butyric acid
JA	:	jasmonic acid
l	:	litre
L/min	:	litre per minute
log	:	logarithm
ln	:	natural logarithm
M	:	molar
m	:	metre

MCF-7	:	Michigan cancer foundation-7
mg	:	milligram
mg/ml	:	milligram per millilitre
mg/L	:	milligram per litre
MeJA	:	methyl jasmonate
MeOH	:	methanol
min	:	minute
ml	:	millilitre
mm	:	millimetre
mM	:	millimolar
MS	:	Murashige and Skoog (1962)
NAA	:	1-Naphthaleneacetic acid
NaCl	:	sodium chloride
NaOH	:	sodium hydroxide
nm	:	nanometre
°C	:	degree celcius
PGR	:	plant growth regulator
pH	:	The negative logarithm of the hydrogen ion concentration
psi	:	pound per square inch
PTFE	:	polytetrafluoroethylene
RAPD	:	Random Amplification of Polymorphic DNA
ROS	:	reactive oxygen species
rpm	:	revolution per minute
RT	:	retention time
R^2	:	square of the correlation coefficient
s	:	second
SA	:	salicylic acid
SCV	:	settle cell volume

SD	:	standard deviation
SE	:	standard error
spp.	:	species
t	:	time
U	:	unit
USA	:	United State of America
USD	:	dollar of United Sates of America
uv	:	ultra violet
v/v	:	volume per volume
vvm	:	volume per volume per minute
viz.	:	Latin : vidalicet or English : namely
w/v	:	weight per volume
ZER	:	zerumbone

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CHAPTER 1: INTRODUCTION

1.1 GENERAL INTRODUCTION

Plants represent an unlimited phytochemical source which includes metabolites of primary and secondary metabolisms. The primary metabolites are important for plant growth, development and reproduction whereas the secondary metabolites may play an essential function in plant protection against herbivores and interspecies defences (Wink, 2003; Li et al., 2016). As for human, plant secondary metabolites are of main interest due to its different functions and their remarkable biological activities, which are useful for medical, pharmaceutical and food applications.

Therefore plants have been extensively used for treating human illness and being linked with human health and culture (Gomez-Galera et al., 2007). Over decades, human have used plant derived compound for treating disease and plant-based remedies have always been an essential part of traditional medicine. Around the world, it is estimated that over 500,000 plant species are used for medicinal purposes and 80 % of worldwide pharmaceutical products are of plant origin. The herbal and medicinal industries have enormous demand worldwide and there has been an increase in the use of novel psycho-active substances, some of which are based on plant natural products (Gibbon & Arunotayanum, 2013). A current report suggests global market for medicinal plants is around $60 billion growing at a brisk pace of 7-11 % annually (Upadhyaya, 2017).

Recently, awareness on the use of plant-based substances for cancer prevention or conventional anticancer therapy had increased. Numerous biologically active substances derived from various plant parts known as phytochemicals have been reported to

contain the cancer preventive potential. Based on this demand, it is expected that the market value of plant-based medical and pharmaceutical products will keep increasing over the coming years.

Although most medicines used in the United States and other developed countries today are based on synthetic pharmaceuticals, there was an increasing trend of cultures around the world in using plant-derived medicines. In US, 38% adults and 12% children are using alternative medicine (Barnes et al., 2008; Vasisht et al., 2016). According to the World Health Organization (WHO), majority of people worldwide use herbal medicine to overcome some of their health care issues. This preference could be due to plant's special nature in boosting immunity beside its inconsequential side effects in comparison to synthetic drugs and antibiotics (Karimi et al., 2015). For example, the use of steroid anti-inflammatory drugs to treat arthritic caused a widespread disturbing adverse effect. Therefore the approach of using herbs to these conditions causes moistening of dry synovia, stimulation of circulation in the affected regions, facilitation of elimination via kidneys and hepatic/biliary routes & dietary modification of metabolism (Kazemipoor et al., 2012).

1.1.1 Medicinal plant industry in Malaysia

Malaysia is one of the most biologically diverse countries and was listed as one of the seventeen countries identified as mega diverse country by Conservation International due to its great heritage of flora and fauna biodiversity (Von Rintelen et al., 2017). As one of the world's richest diversity, it contains diversified flora and fauna with an estimated 12,500 species of flowering plants and 185,000 species of fauna endemic to tropical forests (Biodiversity in Malaysia, 2006). The flowering plants ranging from valuable timber, marketable fruit, and other plants that are identified to

produce natural products, in general regarded as secondary metabolites not only confer adaptive benefits on the plants itself, but also beneficial to human (Noor et al., 2011). For that reason, most of the important pharmaceuticals are derived from plants, and other plant secondary metabolites were used as flavors, dyes, and essential oils that have been treasured by human societies for millennia.

Malaysian tropical rainforest has a huge potential to be explored due to its rich biodiversity in medicinal plants. The Malaysian medicinal and pharmaceutical industry increased from USD1.22 billion in 2009 to USD 1.40 billion in 2010 (Biospectrum, 2011). In urban Malaysia, the consumer demand for traditional medicinal product is high (70%) indicating that the herbal or traditional medicine are still widely accepted as a supplementary health care alongside the modern medical health care system (Hassali et al., 2012; Sooi et al., 2013). The effectiveness and popularity of the traditional medicinal products depend mainly on the anecdotal experience and ethnic belief of the multi-ethnic Malaysian society. The practise of traditional medicine is common among Malaysian ethnic groups such as Malay, Chinese, Indian and aborigines with the knowledge being passed down through the generations.

It is estimated about 20% (3,000 spesies) of angiosperm and gymnosperm plant species found in Malaysia contain medicinal properties and have been used in traditional medicine preparation (Ibrahim, 2004). Medicinal plants commonly belongs to family Anonaceae, Apocynaceae, Araceae, Compositae, Dioscoreaceae, Ebenaceae, Euphorbiaceae, Lauraceae, Myrtaceae, Rutaceae, Zingiberaceae and some others (Soepadmo, 1995). Table 1 displayed the number of genera and medicinal plant species from selected family commonly used in the preparation of traditional medicine.

Table 1.1: Number of genera and plant species from selected family groups used in traditional medicinal preparation.

Family	Number of genera	Number of species
Anonaceae	38	198
Apocynaceae	35	125
Euphorbiaceae	69	370
Lauraceae	16	213
Leguminosae	70	270
Myrsinaceae	9	108
Myrtaceae	9	209
Rubiaceae	80	555
Zingiberaceae	20	140

(Source: Soepadmo, 1995)

Despite the huge market potential for medicinal plants in Malaysia, the Malaysian biodiversity is facing threat of being replaced by mono-specific tree crop such as rubber and oil palm. This is in addition to intensive logging or conversion to non-forestry land for the development of hydroelectric dams and rural settlement. A strategic plan needs to be implemented by the authorities for urbanization without major disturbance to our natural resources conservation. Conservation of our plant biodiversity is crucial for plant breeding programs meant for improvement of food production and new food supply. Without a doubt, such diversity is an imperative and precious national asset to be protected for present and future generations (Noor et al., 2011).

1.1.2 Medicinal plant research in Malaysia

Malaysian biodiversity with enormous plant species provides an opportunity for intensive research in medicinal plant properties. Early studies in medicinal plant research involved the ethno-botany and traditional usage of plants as well as the documentation of poisonous and economically-important plants.

With the establishment of public universities and research institutes in Malaysia, the plant-based medical research is progressively pursued in order to explore plant bioactive properties. Various local medicinal plant species have been investigated for their medicinal properties for instance an anti-inflammatory, antimicrobial, antioxidant, antihypertensive, anti-dengue and anticancer. The scope of research includes herbal and traditional medicine, phytomedicine, pharmaceutical, nutraceutical and essential oils (Maziah et al., 2003). A more recent aspect of research is on the synthesis of biologically active compounds through biotechnology that involved the production of natural products and enzymes from plant cell cultures.

For Malaysian medicinal plant research, the important plants being investigated are based on National Key Economic Area (NKEA) for Agriculture 2013. Research on medicinal plants in Malaysia is focusing on halia (*Zingiber officinale*), tongkat ali (*Eurycoma longifolia*), pegaga (*Centella asiatica*), mengkudu (*Morinda citrifolia*), kunyit (*Cucurma domestica*), hempedu bumi (*Andrographis paniculata*), lengkuas (*Alpinia galangal*), sireh (*Piper betel*), misai kucing (*Orthosiphon stamineus*) and kacip Fatimah (*Labisia pumila).*

1.2 SPECIES STUDIED (*ZINGIBER ZERUMBET* SMITH)

The family Zingiberaceae is a moderately large genus of herbs with 50 genera and represented by approximately 1500 species that are mainly distributed in Asia. Zingiberaceae was believed to be originated from the Indo-Malayan region and is now widely distributed across the tropics of Asia, Australia, Africa and America. The name *Zingiber* was in fact derived from a Sanskrit word meaning bull's horn (Larsen et al., 1999).

Members of the Zingiberaceae family are normally used as a spice for over 2000 years (Bartley & Jacobs, 2000) and contain characteristic odour and flavour such as the pungent taste and smell (Larsen et al., 1999; Jolad et al, 2005). A particular plant belongs to this genus that has gained a lot of interest due to its high medicinal values is *Zingiber zerumbet* (L.) Smith (Yob et al., 2011).

Z. zerumbet or commonly known as Lempoyang is a monocotyledonous herbaceous perennial plant used in traditional medicine, food flavouring and as an appetizer in a variety of cuisines. The rhizome extracts contain bioactive compounds that have widely been used by practitioners in herbal medicine for treating stomach ache, edema, sores, and loss of appetite. Meanwhile, the boiled rhizome juice is used to treat children suffered from worm infestation.

There are many common names for *Z. zerumbet*, which also known as pinecone ginger based on the flower shape. In Malaysia, it is commonly known as Lempoyang in Malaysia and called by other names around at other region of the world such as Krathue (Thailand), Awapuhi kuwahiwi (Hawaii), Barik or langkawas (Philippine), Lampuyang (Indonesia), "*Jangli adha*" (Bangladesh), *Ghatian*", "*Yaiimu*" (India), "*Hong qiu jiang*" (China), "*Zurunbah*"(Arab), "*Awapuhi*" (Hawaii) and Bitter ginger (North American). *Z. zerumbet* is also popularly known by the Hawaiians as shampoo ginger due to its creamy mucilaginous substance presents in the inflorescence, which is used as shampoo as well as natural hair conditioner (Nalawade et al., 2003).

1.2.1 Morphological description

Zingiber zerumbet is a perennial edible ginger with stems of approximately 1-2 m tall. The leaves and inflorescences of the pinecone ginger emerged from a thick knobbly underground rhizome that grows just under the surface of the soil. It has an arching pseudostem with blade-shaped leaves measuring 15-20 cm long, arranged alternately (Yob et al., 2011). After the leafy stems have been growing for awhile, the conical or club-shaped flower heads burst forth on separate and shorter stalks hidden beneath the leaf stalks (Figure 1.1A).

The flower heads are initially green and turned to reddish-green after a period of time. The flower head measured 3-10 cm long possesses overlapping scales, enclosing small yellowish-white flowers that poke out a few at a time (Figure 1.1B). The yellowish-white flowers which are usually longer than bracts, produced around August to September are fragile, short-lived and only lasting a few hours. As the flower heads mature, they turn into a brighter red colour and gradually fill with slimy aromatic liquid.

The rhizome of *Z. zerumbet*, which is pale yellow in colour are perennial, thick, scaly with pungent aroma (Figure 1.1C-D), are the asexual reproducer for the species propagation. From single rhizome, it can grow into a large clump within a year and propagation is by the division of clump or pieces of rhizome at a new cultivation plot.

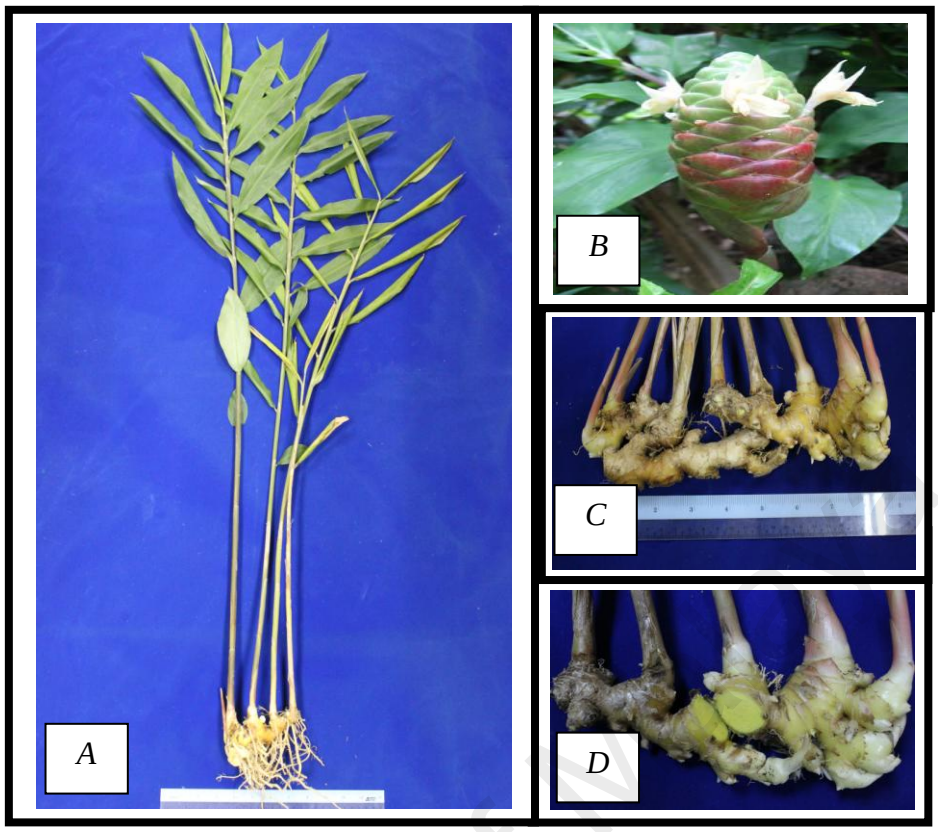

Figure 1.1: *Zingiber zerumbet* plants, *A*: whole plant of *Z. zerumbet*, *B*: inflorescence, *C*: rhizomes, *D*: pale yellow rhizome discoloration

1.2.2 Common uses

Almost all parts of *Z. zerumbet* plant have its special usage from ornamental, personal care, food flavouring to ethno medical practice. In general, the plant's pine cones are used for indoor or outdoor decorative purposes such as in flower arrangement and landscape. As for the milky juice obtained from the pine cones, it is used as a shampoo particularly in Hawaii.

Z. zerumbet is believed to be native to India and the Malay Peninsula. This corroborates with the use of its rhizome as food flavouring and appetizer in Malay and Indian cuisines. The East Malaysian people use the young leaf of *Z. zerumbet* to prepare their local appetizer called 'kerabu Lempoyang'. In Malaysia and Indonesia, the rhizome is sliced and added to food prepared for confinement women. The rhizome

could also been dried and pulverized to be added as one of the ingredients in 'jamu' making for the wellbeing and rejuvenating beauty of confinement women.

In spite of its regular uses as food flavouring and appetizer, the rhizomes of *Z. zerumbet*, in particular, have also been used in ethnomedicine to treat various ailments (Norulaini et al., 2009; Sulaiman et al., 2010). Generally, the rhizome's traditional usages as ethnomedicine include the treatment of fever, indigestion, toothache, inflammation, diarrhea, constipation and severe sprains. It also serves to relieve pain, as well as antirheumatic, antispasmodic and diuretic agents (Bhuiyan et al., 2009; Zakaria et al., 2010; Sulaiman et al., 2010).

The Malays ethnic used the fresh rhizomes as infusion with water to treat stomach ache while the juice of the boiled rhizome is used to treat worm infestation in children. The infusion-salt mixture could be used to cure injury from a bump to the head and applied to skin for ringworm treatment in child. Meanwhile, alcohol-macerated rhizomes are use as tonic, depurative, or stimulant in Chinese traditional medicine practise. As for the Taiwanese, they use the plant as an anti-inflammatory adjuvant especially for stomach ache, fever and sprain. In India, the mixture of rhizome powder with ripe *Morinda citrifolia* is prepared for severe pain treatment. While, the boiled and softened rhizome is used to treat various ailments from cough, asthma, toothache, worms infestation and to a certain extend in treating contagious disease such as leprosy, and other skin diseases.

Other usage of *Z. zerumbet* plants by the Hawaiians is to treat headache, toothache, stomach-ache, ringworm/other skin disease and achy joints/sprains. For treating headache, the rhizome was crushed with salt and rubbed onto the treated area. In

addition, a few approaches was used in treating wound and bruises such as by applying the compressed rhizome onto wounded area or use of ashes from burnt leaves of *Z. zerumbet*, which are combined with a mixture of ashes of *Schizostachyum glaucifolium*, nutsap of *Aleurites moluccana*, and tuber sap of *Z. zerumbet*, as a remedy for cuts and bruised skin (Chun, 1994).

1.2.3 Phytochemical contents

The bioactive compounds in *Z. zerumbet* are normally found in the essential oil and it can be extracted from all parts of the plant particularly the rhizomes, leaves and flowers (Chane-Ming et al., 2003). Leaves and flowers give different type of bioactive compounds than rhizome with the major ones are (*E*)-nerolidol, followed by beta-cryophyllene and linalool. Dung et al. (1993, 1995) reported on high proportions of (*Z*)-nerolidol (22-36%) in the extracts of all parts of the plant except the rhizomes. The leaves and flowers also contain zingiberene (Bhuiyan et al., 2009). The presence of zerumbone (37%) was reported as the major compound in rhizome of *Z. zerumbet* in almost all the studies done on rhizome extract followed by alpha-humulene (14%) and camphene (13.8%).

Due to the differences in chemical content, the rhizome has been the focus of extensive research due to its high phytomedicinal values. Various reports have been published on the phytochemical content of *Z. zerumbet* rhizome. Earlier studies involved the identification of bioactive compounds such as humulene, monoterpenes and zerumbone (2,6,10-cy-cloundecatrien-1-one, 2,6,9,9-tetramethyl-,(*E*,*E*,*E*)-) from the essential oil of *Z. zerumbet* rhizome. Further phytochemical investigations on rhizome of *Z. zerumbet* showed evidence of the presence of sesquiterpenoids, flavonoids, aromatic compounds (e.g. hydroxyl benzaldehyde), vanillin and kaempferol derivatives

(Dae et al., 2004; Lako et al., 2007, Chien et al., 2008). Srivastava et al. (2000) and Yu et al. (2008) also revealed the occurrence of approximately 86% sesquiterpenoids in the essential oil of *Z. zerumbet* with zerumbone being the major component. Other compounds present are *β*-caryophyllene, caryophyllene oxide and *β*-eudesmol. While Chane-Ming et al. (2003) reported that the essential oil of *Z. zerumbet* was affluent in zerumbone, *α*-pinene, and camphene.

1.2.4 Pharmacological properties

The Zingiberaceae species are well known as perennial herbs rich in potential bioactive compounds of phytomedical interest. Previous studies confirmed the diverse pharmacological prospective of *Z. zerumbet* in a range of *in vitro* and *in vivo* test models (Yob et al., 2011). The rhizome of *Z. zerumbet* has specifically been verified to possess antiallergic, antihyperglycemic, anti-inflammatory, antimicrobial, antioxidant, antiplatelet, antiulcer and anticancer activities at different doses or concentrations.

Recent finding has proved that the rhizome of *Z. zerumbet* contained a novel bioactive compound with suppressive effect known as zerumbone. Zerumbone is potentially useful in chemoprevention, whereby it was found to inhibit Epstein-Bar virus (EBV), a herpes virus latently affecting human *b*-lymphocytes which was believed to be contributing factor in some human cancers (Murakami et al., 1999). Further studies showed that zerumbone compound is able to suppress tumor promoter cells (Murakami et al., 2002). This was supported by Huang et al. (2005) where they reported that zerumbone inhibited the growth of human leukemia cell line, HL-60. Zerumbone has also been shown to exhibit antiproliferative effects on different cancer cells lines namely HT-29, CaCo-2 and HepG2 cancer cells (Chien et al., 2008; Kirana et al., 2003). More recent study done on zerumbone showed that it possesses antiproliferative

properties against HeLa (cervix), MCF-7 (breast) and Coav-3 (ovary) human cancer cells and at the same time exerts less effect on normal cells of CHO (Wahab et al., 2009). With intense cytotoxic effects against cancer cells in comparison to normal cells, zerumbone would have a bright future and could be serve as a promising bioactive compound in tumour and cancer research.

1.3 SCOPE OF RESEARCH

Zerumbone compounds will be identified from cell suspension and adventitious root cultures. As an alternative source for this compound, cell suspension and adventitious root cultures will be established with defined formulation and conditions for biomass growth and accumulation of zerumbone. The enhancement of zerumbone production by cell and adventitious root cultures will be made through manipulation of physical and chemical factors such as medium strength, initial inoculation volume, initial pH, agitation, culture temperature, carbon sources and concentration and type of plant growth regulators (PGRs).

Application of *in vitro* cultures in scaled-up production of both biomass and targeted metabolites in balloon-type bioreactor was investigated. The effects of selected parameters such as air flowrate, initial inoculum volume and pH on the *in vitro* cultures were studied. The effects of elicitors such as methyl jasmonate (MeJA) and salicylic acid (SA) on zerumbone production in adventitious roots culture will be investigated. The relationship between zerumbone production and selected enzymes that detoxify reactive oxygen species (ROS) will also be studied. The selected enzymes are hydrogen peroxide, catalase (CAT) and ascorbate peroxidase (APX).

General objective:

To establish *in vitro* biomass production of *Zingiber zerumbet* and to develop enhancement strategies for zerumbone accumulation in cell suspension and adventitious root cultures in shake flasks and controlled bioreactors.

Specific objective:

1. To optimize the growth of cell suspension and adventitious root cultures of *Z. zerumbet* in shake flask system through manipulation of selected chemical and physical parameters;
2. To evaluate the production of zerumbone in shake flask system by *Z. zerumbet* cells suspension and adventitious root cultures;
3. To study the biomass growth of adventitious root cultures in controlled bioreactor and its accumulation of zerumbone;
4. To enhance zerumbone production of adventitious root culture through chemical elicitation;
5. To investigate the correlation between zerumbone production and selected enzymes that detoxifies reactive oxygen species (ROS).

Hypothesis:

Systematic manipulation of selected physical and chemical factors could help to enhance the biomass growth and accumulation of zerumbone in cell suspension and adventitious root cultures of *Z. zerumbet* Smith.

CHAPTER 2: LITERATURE REVIEW

2.1 PROPAGATION OF *ZINGIBER ZERUMBET* SMITH

2.1.1 Conventional propagation

Zingiber zerumbet is conventionally propagated by using rhizome due to the lack of seedset in Zingiberaceae family (Koga et al., 2016). For Zingiberaceae planting, growers generally use part of the rhizome from previous harvest called seed-pieces as planting stock (Smith & Hamill, 1996). This practice gave high chances of escalating the possibility of spreading endogenous diseases as well as contends with human consumption for rhizomes usage, thus making the establishment of large scale ginger plantations a difficulty (Villamor, 2012).

The conventional method also permits transmission of soil-borne pathogen and spreading of diseases either to other plants or planting areas. The disease could be transmitted through different ways including planting infected rhizomes (Hayward & Pegg, 2013). The *Zingiber* species are susceptible to soil-borne diseases such as rhizome soft rot and leaf spot diseases (Meenu et al., 2017). It is also prone to soil-borne pathogen such as *Colletotrichum* species (Chan, 2004). Currently the most serious diseases are root-knot nematodes (eg. *Meloidogyne incognita*, *M. javanica*) and *Fusarium* yellows (eg. *Fusarium oxysporum* f.sp. *zingiber*). Their effect on crop yields can be greatly exacerbated when infested planting materials are used. Nematode-infested seed pieces could result in high yield loss even if planted on fumigated soil (Okorocha et al., 2014). Another limitation of conventional propagation is the requirement of proper maintenance and preservation of seed-pieces during the dormancy phase before the next replanting process to prevent *Fusarium* infection (Le et al., 2014). This could destroy rhizome tissues during storage and readily infect plants during all stages of development (Melati et al., 2016).

The major constraint in the Zingiberaceae cultivation is its low productivity caused by deprived genetic stock and occurrence of pests and diseases due to conventional planting practice (Nalawade et al., 2003). It is necessary to develop a suitable method of propagation through biotechnology to produce disease-free and high quality planting materials. Since the conventional clonal material causes disease build-up, which drastically reduces crop yield, *in vitro* propagation is usually favoured. Plant propagation through *in vitro* regeneration is a simple and cost-effective method to produce clonal planting materials within a relatively short period of time (Zuraida et al., 2016).

2.1.2 *In vitro* culture technique

2.1.2.1 Micropropagation

Micropropagation is an advanced technique of regenerating plantlets by using explants proliferated on formulated semisolid or in liquid media under aseptic and controlled condition (Loyola-Vargas et al., 2008). This *in vitro* technique offers the possibility of rapid clonal multiplication of elite plant species, allowing production of genetically stable and true-to type progeny (Barakat, 2008). The key factor for micropropagation lies on the ability of fast clonal multiplication and production of disease-free plants (Hrahsel & Thangjam, 2013). This technique has been employed as propagation tool in many plant species and is increasingly essential for commercial production. It has developed into a preferred method of cloning and bulking as well as enhance crop production and to aid crop improvement (George, 2003).

Zingiberaceae is traditionally propagated by rhizome which can only be stored for a limited period of time, as it is at risk of fungal diseases and thus will affect the quality of the rhizomes (Nalawade et al., 2003). Thus, micropropagation can be used as an

alternative method for producing pathogen-free plants in a large amount with regards to homogeneity and good quality rhizomes (Poonsapaya & Kraisintu, 1993). There are five major phases in plant micropropagation including selection of suitable planting material, establishment of sterilization procedure for explant, initiation of explant on suitable medium under aseptic condition, multiplication of shoots and rooting of plantlets and finally acclimatization of *in vitro* plantlets prior transferring to soil.

Studies on *in vitro* tissue culture in *Zingiberaceae* spp. started in late 70's with low survival rate in field planting (Faridah et al., 2011). Over the years, there are extensive work carried out with respect to micropropagation (Bhagyalakshmi & Singh, 1988; Noguchi & Yamakawa, 1988; Wang, 1989; Vincent et al., 1992, Faria & Illg, 1995; Smith & Hamil, 1996; Borthakur et al., 1999; Poonsapaya & Kraisintu, 1993; Rao & Das, 1997), plant regeneration by callus organogenesis (Malamug et al., 1991), somatic embryogenesis (Babu et al., 1992; Kackar et al., 1993), *in vitro* germplasm conservation (Dekkers et al., 1991), production of disease-free encapsulated buds (Sharma et al., 1994), production of microrhizomes (Sharma & Singh, 1995) and disease indexing on *in vitro* propagated rhizome (Sharma & Singh, 1997).

In the new millenium, micropropagation of commercial and endangered Zingiberaceae species have been carried out (Khatun et al., 2003; Chan et al., 2004; Loc et al., 2005; Bharalee et al., 2005, Bejoy et al., 2006., Stanley & Kang, 2007; Yusuf et al., 2007; Faridah et al., 2011; Yusuf et al., 2011). Other works on enhancement of propagation using callus organogenesis (Salvi et al., 2001), somatic embryogenesis (Tan et al., 2005; Idris et al., 2010) and microrhizome (Nayak & Naik, 2006; Anisuzzaman et al., 2008; Zheng et al., 2008; Archana et al., 2013a,b; Zuraida et al., 2015; Swarnathilaka et al., 2016) were also reported. Observation on field

performance of *in vitro* plantlets, which includes the study on variation and genetic stability by using RAPD was done for various species (Salvi et al., 2002; Panda et al., 2007; Das et al., 2010; Siju et al., 2010; Mohanty et al., 2011).

Nowadays, with the advancement in biotechnology and the discovery of potential bioactive compounds in Zingiberaceace (Tan et al., 2006; Kirana et al., 2007), new prospect of study were explored by using cell culture to extract bioactive compounds from Zingiberaceae cell suspension (Yusuf et al., 2013; Jalil et al., 2015) and root culture (Raju et al., 2015).

2.1.2.2 Callus culture

Plants produce unorganized cell masses called callus, in response to stress, such as wounding or pathogen infection. The term callus derives from the Latin word "callum", which means hard. In the beginning of plant biology, the term "callus" referred to the enormous cells growth (callose) that associated with wounding. The formation of wound-induced callus has long been observed and employed in a range of perspectives from debarking of trees (Stobbe et al., 2002) to horticultural use of propagation (Momoko et al., 2013). These calli was derived from various types of cell, which include cortical, vascular and pith cells. In several cases, wound-induced calli could regenerate new organs or tissues, implying that they are highly pluripotent (Stobbe et al., 2002). Collin and Edwards (1998) referred callus as a response to wound from any original plant parts ranging from the common plant organs (e.g. leaves, petioles, stems and roots) to the specific plant tissue or cells (e.g. mesophyll, pollen and endosperm).

Street (1997) define a callus culture as an unorganized plant tissue growing on a solidified medium while Sugimoto et al. (2011) referred it as the initial undifferentiated growth obtained from the explant upon transfer to the agar containing nutrients. The phenomenon of capability of living cells giving rise to full plants is called cellular totipotency (Bhojwani, 1983). In general, most of the callus cells are totipotent and thus has the ability to regenerate the whole plant body (Sugimoto et al., 2011). The induction of callus in various plant species could be achieved by application of auxin and cytokinin exogenously with an intermediate ratio of both plant growth regulators. While a high ratio of auxin-to-cytokinin or cytokinin-to-auxin induces root and shoot regeneration, respectively. For a certain cases, other hormones may substitute auxin or cytokinin in callus induction, e.g. abscisic acid and brassinosteroid (Hu et al., 2000). Nonetheless, auxin and cytokinin have been considerably the most widely used and studied plant growth regulators in the context of callus formation and subsequent organ regeneration (Ikeuchi et al., 2013).

Callus is maintained through subculture, by transferring the newly formed callus tissue from the explants to a new agar medium periodically and the time frame is dependent on the plant variety (Carsono et al., 2006). The new growth of callus tissue formed on the outside of the transferred callus results in heterogeneous accumulation of old and young cells (Samariya & Sarin, 2014). The success of callus initiation depends on several factors such as medium components, culture conditions, explant types and plant genotypes (Nakasha et al., 2016, Al-Khayri et al., 2017). The age and physiological state of the mother plant could also affect the callus formation. Any parts of plants including mature leaves, roots, stem, flowers and seeds can be used for callus initiation, but the preferred explant materials are younger meristematic and fresh explant tissue. Compared to monocots, dicots are rather acquiescent in callus formation

whilst callus of woody plants are generally slow in growth (Street, 1997).

Transfer of callus tissue onto differentiation medium results in shoot or root regeneration and under certain conditions, it could produce somatic embryo. Somatic embryo refers to embryos which are generated from somatic cells. Recently, several studies have revealed that ectopic over-expression of meristematic or embryonic regulators induced the formation of callus in various plant species (Wang et al., 2011). The formation of calli is through the modulation of plant hormone signaling, particularly of auxin and cytokinin. The hormone signaling pathways involved several key regulators which functioned during callus induction (e.g., ARFs and ARRs) (Ikeuchi et al., 2013). It is also believed that the intrinsic developmental programs, such as embryogenesis and meristem formation are used in callus formation. Under normal growth conditions, these programs are spatially and temporally restricted but after experiencing certain environmental challenges it become ectopically activated. It is possibly due to the fact that these hormonal and its developmental pathways are inter-connected at multiple levels (Ikeuchi et al., 2013). The developmental and differentiation of embryonic and meristematic programs also involved the epigenetic repression. Epigenetic regulators such as chromatin modification, which include DNA methylation and histone modification, affect gene expression. Epigenetic regulators also regulate global chromatin status which plays vital role in the control of cell differentiation and dedifferentiation (Gaspar-Maia et al., 2011; Grafi et al., 2011).

Callus is very diverse and can be classified into various types based on their macroscopic characteristics. Callus with lack of organ regeneration normally are known as friable or compact callus. While, callus that exhibit organ regeneration are called organogenic, or embryonic callus, depending on the organs generated which

undoubtedly related to gene expression distinctive profiles (Iwase et al., 2011). To date, callus cultures are an essential tool in plant biotechnology. For Zingiberaceae species, it has been shown that shoot organogenesis *via* an intermediate callus phase served as an effective method for multiplication (Malamug et al., 1991; Kackar et al., 1993; Salvi et al., 2001; Tan et al., 2005). Besides the common usage of the regeneration system, calli is use to enhance economically important traits in plants by the introduction of transgenes through plant transformation.

2.1.2.3 Cell suspension culture

Initiation of cell suspension cultures starts by transferring a small portion of undifferentiated callus into a liquid medium, which is then agitated during the culture period. The agitation of the culture is important to break the callus and to prevent the accumulation of large clumps of cells. Generally, a suspension culture consists of a mixture of single cells, small cell groups and large cell aggregates, dispersed in a liquid medium. Cells suspension consists of actively growing cells, which divide under agitation and aeration to increase the cell biomass. Walton et al. (1999) stated that the ideal cell suspension cultures should consist of only single cells but, in reality, they contain range of cell aggregates, some containing up to several hundred cells. Upon transferring of the callus into the liquid medium, the cell suspension enter an initial lag phase prior to any sign of cell division followed by an exponential rise in cell number and a linear increase in the cell density. Lastly, the cells enter a stationary or non-dividing stage due to a gradual deceleration in the division rate. To maintain the viability of cells, subculturing of the cell masses within a certain period is critical and dependent on the doubling time of the cell cultures (Heinstein & Emery, 1988).

Cell suspension cultures are extensively used in plant biotechnology as a convenient tool for propagation and plant improvement. To date, cell suspension cultures have been employed for many plant species ranging from model systems like *Arabidopsis*, *Catharanthus* and *Taxus*, to important crop plants such as rice, soya bean, alfalfa, banana, tobacco, potato and medicinal plants (Zhang et al., 2000; Jalil et al., 2003; Jeong et al., 2008; Torabi et al., 2008; Fan-Suo et al., 2009; Wong et al., 2013; Vijaya Sree et al., 2010; Yusuf et al., 2013; Karwasara & Dixit 2013; Ahmad & Baig, 2014; Wang et al., 2015; Deepthi & Satheeshkumar, 2016)

Boisson et al. (2012) stated that suspension culture of isolated plant cells is an important tool in providing the material for high-throughput studies such as metabolic analyses, herbicide discovery and secondary plant compounds production. The homogenous population of cells enables easy physiological and biochemical experimentation. The homogeneity of an *in vitro* cell population, large availability of material, high growth rate and reproducibility of cells enable its usage for complex physiological analysis at the cellular and molecular levels. Furthermore, plant cell cultures offer a valuable platform for the production of high-value secondary metabolites and other compounds of commercial importance (Moscatiello et al., 2013). Harvesting of compounds from cell suspension culture offers many advantages including:

i) multiplication of any type of plant cells to produce bioactive compounds
ii) production of useful bioactive compounds under controlled conditions to meet market demands
iii) assurance of consistent product quality with the use of specific cell lines
iv) providing a continuous and reliable source of bioactive compounds

This is the preferred type of culture for large-scale production because of its similarity of rapid growth cycle to microbial cultures. Walton et al. (1999) discussed the ease of using suspension cultures in compound extraction since the growth rates are greatly higher than callus culture owing to the extensive growth of young meristematic cells.

For naturally occurring secondary metabolites such as shikonin, anthocyanins, flavonoids, ajmalicine, terpenoids and imperative anti-tumor compounds like taxol, vinblastine and vincristine (Oksman-Caldentey & Inze, 2004, Saiman et al., 2014), cell suspension cultures were successfully established. A well-established cell suspension system permits rapid enhancement of desired compounds production through metabolic engineering and chemical elicitation (Yusuf et al., 2013). Two important steps required for efficient production of bioactive compounds via cells suspension culture are:

(i) The initiation of fast and extensive growth of cells in large volumes, which are subsequently manipulated to produce secondary metabolites;

(ii) The growth and subsequent immobilization of cells, which are used for the production of bioactive compounds over a prolonged period.

2.1.2.4 Root culture

In vitro bioactive compounds are produced either from undifferentiated or differentiated cells. The most commonly used differentiated cell cultures are adventitious and hairy roots due to its rapid growth and the ability of large-scale cultivation in bioreactors. These compounds are used as pharmaceuticals, pigments and flavours.

Adventitious root (AdR) culture is induced by plant growth regulators (PGRs) which respond to mechanical damage of explant during tissue culture activity whereas *Agrobacterium rhizogenes* causes hairy root disease in plants. There are two pathways by which AdR forms or can be induced to form. Firstly, through direct organogenesis from established cell types such as cambium, or secondly, from callus tissue following mechanical injury during explant excision. AdR formation involves redifferentiation process, by which predetermined cells switch from their morphogenic pathway to act as mother cells for the root primodia.

The process of of obtaining adventitious root consists of three suscessive interdependent physiological phases each with different requirements namely, induction (includes molecular and biochemical events without discernible changes), initiation (characterized by cell divisions and root primodia organization) and expression (characterized by intra-stem growth of root-primodia and root emergance) (Schwambach et al., 2005). Several environmental as well as endogenous factors, for example hormones (especially auxin), sugars, mineral salts, temperature and light conditions may function as signals and induce groups of cells to redefine their fate, resulting in and regulating AdR. Phytohormones play complex roles in exerting direct (acting on cell division or cell growth) or indirect (interacting with other molecules or phytohormones) effect (Sarin, 2005). As for hairy roots, the neoplastic (cancerous) roots produced by *Agrobacterium rhizogenes* infection are characterized by propagation in hormone-free growth media, high growth rate and genetic stability. The bioactive compounds produced by these genetically transformed roots are comparable intact plants.

Compared to hairy root cultures and field cultivation, adventitious root approach is safer, more stable and easier for compound extraction management (Sudha & Seeni, 2001). Adventitious roots initiated through *in vitro* methods displayed high proliferation rate and active secondary metabolism (Yu et al., 2005). The ability to form adventitious roots is a heritable quantitative trait controlled by multiple endogenous and environmental factors, among which the plant hormone auxin plays a vital role (Geiss et al., 2009; Li et al., 2009). Nontheless, hairy root cultures also offer a promise for high production and productivity of valuable bioactive compounds used as pharmaceuticals, pigments and flavors in many plants species (Srivastava & Srivastava, 2007).

The adventitious root cultures induced by plant growth regulators (PGRs) have been studied in various medicinal plants species including *Withania somnifera* (Rani et al., 2003a), *Panax ginseng* (Paek et al., 2005), *Echinacea angustifolia* (Wu et al., 2006), *Andrographis paniculata* (Praveen et al., 2009b), *Hypericum perforatum* (Cui et al. 2010a), *Stevia rebaudiana* (Reis et al., 2011), and *Morinda citrifolia* (Baque et al., 2010a,b,c), *Gynura procumbens* (Saiman et al., 2012), *Boerhaavia diffusa* (Jenifer et al., 2012), *Psammosilene tunicoides* (Zhang et al., 2013), *Rumex crispus* (Mahdieh et al., 2015), *Plumbago rosea* (Silja & Satheeshkumar, 2015), *Passiflora pohlii* (Simão et al., 2016) and *Couroupita guianensis* (Manokari & Shekhawat, 2016). Bioactive compound harvesting using hairy root cultures have been successful in many plant species such as *Cantharanthus roseous* (Toivonen et al., 1989; Vázquez-Flota et al., 1994), *Gingko biloba* (Shunan et al., 1997), *Datura stramonium* (Baiza et al., 1998), *Datura metel* (Cusido et al., 1999), *Artesimia annua* (Xie et al., 2000), *Physalis minima* (Azlan et al., 2002), *Panax ginseng* (Yu et al., 2005a), *Podophyllum peltatum* (Anbazhagan et al., 2008), *Atropa belladonna* (Yang et al., 2011), *Boerhaavia diffusa* (Jenifer et al., 2008), *Nicotiana tabacum* (Zhao et al., 2013), *Silybum marianum* (Hasanloo et al.,

2013), *Taxus x media* var *Hicksii* (Syklowska-Baranek et al., 2014), *Cannabis sativa* (Farag & Kayser, 2015) and *Tagetes erecta* (Gupta et al., 2016).

2.2 PLANT SECONDARY METABOLITES

Secondary metabolites are biosynthetically derived from primary plant metabolism (Sarin, 2005). Even though secondary metabolites are not involved in metabolic activity, it is of great importance for plant to survive in its environment (Wink, 2006; Tiwari & Rana, 2015). Plant secondary metabolites or commonly known as secondary compounds consists of alkaloids, phenolics, sterol, steroids, essential oil, tannin, terpenoid, flavonoids and many others (Sarin, 2005).

Secondary metabolites are of low molecular weight and not required for normal growth and development of plant. There is no common metabolic pathway for these metabolites in all plants and their occurrence is restricted to special plant families or genera (Alfermann et al., 1995). These secondary compounds are usually synthesized in specialized cells and at a particular developmental stage. Even though the accumulation of secondary metabolites is lower than the primary metabolites, their role in defense and protection against biotic and abiotic stress, as well as attractants to pollinators in terms of colour, aroma and for seed dispersal (Verpoorte & Memelink, 2002; Ramawat, 2007).

For human purposes, these compounds are used as pharmaceuticals, agrochemicals, food additives, pesticides, flavor and fragrance ingredients (Verpoorte et al., 2000; Verpoorte & Hoopen, 2006; Vijaya Sree et al., 2010). These secondary metabolites has antioxidant properties which act as defensive compounds in the human body by increasing the immune system thus giving protection to the body from free radicals (Cowan, 1999; Verpoorte & Memelink, 2002).

Plants containing bioactive compounds used in medicine or prophylactic purpose are commonly known as medicinal plants. For the majority of global population, medicinal plants are the most exclusive source of life saving drugs. The usage of medicinal plants are mainly as crude extracts while some of the more powerful and active substances are employed as isolated compounds as shown in Table 2.1.

Table 2.1: Important plant derived pharmaceuticals.

Secondary metabolites	**Use**	**Plant species**
Ajmalicine	Antihypertensive	*Catharanthus roseus*
Artemisinin	Antimalarial	*Artemisia annua*
Berberine	Intestinal ailment	*Coptis japonica*
Camptothecine	Antitumor	*Camptotheca acuminate*
Capsaicin	Counterirritant	*Capsicum frutescens*
Castanopermine	Glycoside inhibitor	*Castanospermum australe*
Codeine	Sedative/Analgesic	*Papaver somniferum*
Colchicine	Antitumor	*Colchium autumnale*
Digoxin	Heart stimulant	*Digitalis lanata*
Diosgenin	Steroidal precursor	*Dioscorea deltoidea*
Ellipticine	Antitumor	*Orchrosia elliptica*
Emetine	Emetic	*Cephaclis ipecaccuanha*
Forskolin	Bronchial asthma	*Coleus forskolii*
Ginsenosides	Heath tonic	*Panax ginseng*
Morphine	Sedative/Analgesic	*Papaver somniferum*
Podophyllotoxin	Antitumor	*Podophyllum petalum*
Quinine	Antimalarial	*Cinchona ledgeriana*
Shikonin	Antibacterial	*Lithospermum erythrorhizon*
Taxol	Anticancer	*Texus brevifolia*
Vincristine	Anticancer	*Catharanthus roseus*
Vinblastine	Anticancer	*Catharanthus roseus*

Source: Ramachandra and Ravishankar (2002)

Over the centuries, the traditional medicine relied on plant secondary metabolites in treating illness and improving human health (Gomez-Galera et al., 2007). Due to historical and cultural reasons, herbal medicines (phytopharmaceuticals) have often maintained popularity in developed countries even though modern medicine is available. In Africa and Asia, about 80 percent of the population still make use of traditional remedies rather than modern medicine for primary healthcare. With the knowledge being passed through oral history from generation to generation, traditional medicine which involved the usage of herbal medicine foresees new development with the growing expertise in biotechnology (Canter et al., 2005). The industry has invested millions of US dollars to uncover promising medicinal herbs and novel chemical compounds (Tilburt & Kaptchuk, 2008). Saklani and Kutty (2008) stated that the global market value for plant-derived drugs was USD18 billion in 2005 and it is estimated to grow up to USD26 billion by 2011.

In Malaysia, extensive research on native medicinal plants was carried out as shown in Table 2.2 whereby some of these compounds have been commercialised. Some of these secondary metabolites are difficult to synthesize chemically, or it is hard to produce or to increase the amount using microorganisms through genetic engineering due to its structural complexity and high cost. The normal practice of secondary metabolites extraction is directly from plants grown in natural habitat but it was hampered by several factors that can alter their yield. Through research in plant biotechnology, the production of many pharmaceutical substances for new therapeutics has been discovered.

Table 2.2: Some example of popular medicinal plants with economic value.

Species	Part (s) used	Treatment
Alpinia galanga	Rhizome	Indigestion, flatulence, colic, dysentery, skin disease
Andrographis paniculata	Leaves	Snake bites, insect stings, fever, diabetes, skin disease, flatulence, antihypertensive
Centella asiatica	Leaves or whole plants	Ulcers, cooling, rheumatism, cough, bronchitis, asthma, gastric catarrh, dysentery
Curcuma domestica	Rhizome	Stomachic stimulant, carminative, treat minor wound, ulcers, abscesses, inflammations, diarrhea,dysentery, flatulence, rheumatism
Curcuma xanthorrhiza	Rhizome	Postpartum medicine, dysentery, bloody diarrhea, infected wounds, treatment for liver affection (jaundice, gall stone and promoting the flow of bile)
Cymbopogon nardus	Leaves, roots	Postpartum bath, a diuretic, treatment of kidneystone, oil used to relieve rheumatic pains
Eurycoma longifolia	Roots	Treatment for fever, wounds, ulcers, tonic for postpartum medicine, decoction to relieve pain in the bones
	Leaves	Decoction to reduce fever, paste for treat dermatitis, wounds and relieve itches
	Bark	Relieve lumbago, treat jaundice, fever, diarrhea, wounds and ulcers
	Fruit	Dysentery
	Stem	Coughs
Labisia pumila	Whole plant	Decoction used before childbirth to expedite labor
	Leaves	Colic
	Roots	Treatment muscle pain, constipation
Morinda citrifolia	Leaves	Heated leaves for treatment of coughs, nausea, abdominal colic, fever
	Fruits	Stomachic, laxative, treatment for diabetes
Orthosiphon stamineus	Leaves	Diuretic, high blood pressure
	Leaves and roots	Decoction for treatment of diabetes
	Whole plant	Decoction for treatment kidney stone
Piper betle	Leaves	Dysentry, bronchitis, rheumatism, chew as stimulant, heated leaves relieve cough and asthma
Zingiber officinale	Rhizome	Diuretic, stimulant, carminative, decoction for treatment of flatulence, antinausea, treatment for cold, rheumatism, cataracts, lumbago, menstrual pain

Source: Ibrahim (2004)

2.2.1 Plant cell culture for secondary compound production

In recent years, with the developing of secondary metabolites for commercial importance, it resulted in great interest in secondary metabolites production through cell culture technology (Vijaya Sree et al., 2010). The usage of plant cell culture has overcome quite a lot of inconveniences for these secondary metabolites production and offers an alternative to agricultural processes in producing valuable phytochemicals. This is because plant cell cultures is not restricted by environmental, ecological or climatic conditions, and thus proliferate at higher growth rates than those of whole plants in cultivation (Zhong, 2001). By using plant cell cultures, controlled growth environment could ensure high quality and quantity production of targeted secondary metabolites. Plant cell cultures could also ensure continuous supply and prevents the possibility of extinction of the wild species (Alfermann & Petersen, 1995; Vijaya Sree et al., 2010).

The main advantage of this technology is that besides providing continuous, reliable source of phytochemicals, it could be extended to large scale production (Vijaya Sree et al., 2010). Plant cell culture may also offer better selectivity and yield for the desired secondary products, since high yielding cell lines could be selected (Misawa, 1994). Besides, plant cell cultures comprise a higher metabolism rate than intact differentiated plants since the growth of cells *in vitro* leads to rapid proliferation of cell mass and to a condensed biosynthetic cycle (Dornenburg & Knorr, 1995).

Nowadays, plant cell culture technology has been successfully applied to various secondary metabolite production including pharmaceuticals, pigments, and other fine chemicals (Verpoorte et al., 2002). The early detection of secondary metabolite from cell culture was in callus cultures of *Andrographis paniculata* for paniculides (Allison *et*

al., 1968). Since then, many other work focusing on the extraction of secondary metabolites produced by cell culture such asterol from *Digitalis lanata* (Halmbold et. al., 1978), shikonin from *Lithospermum erythrorhizon* (Fujita et al., 1981), berberine from *Coptis japonica* (Sato & Yamada, 1984), paclitaxel from *Taxus brevifolia* (Kim et al., 1995), rosmarinic acid from *Coleus blumei* (Petersen et al., 1995), chlorogenic acid from *Eucommia ulmoides* (Wang et al., 2003), ginsenoside from *Panax ginseng* (Sivakumar et al., 2005), isocamptothecin A and B from *Camphotheca acuminata* (Yu et al., 2005), ajmalicine and serpentine from *Catharanthus roseus* (Zhao & Verpoorte, 2007) and podophyllotoxin from *Podophyllum peltatum* (Anbazhagan et al., 2008).

For the purpose of commercialization, cell culture production was scaled-up in bioreactor. Nevertheless, only a few cell culture-induced secondary metabolites were successfully commercialized by industries namely shikonin and barberine by Mitsui Petrochemical Industry (Japan), paclitaxel from Taxus spp. by Phyton Biotech Inc. (Germany) and ginsenosides from *Panax ginseng* by Nitto Denko Corp. (Japan) and CBN Biotech Company in South Korea (Zhao & Verpoorte, 2007; Baque et al., 2012b).

2.2.2 Sources of secondary metabolites through conventional approach

The general practise for secondary metabolite extraction in industry is by harvesting the field grown plants. This needs abundant and consistent qualities of planting materials to enable continuous supply of quality bioactive compounds for commercialization. The current practice of harvesting plant materials for bioactive compound needs a long period of plant cultivation which faces uncontrolled climatic conditions. For example, in commercial production of hypericin from field-grown plant material, the quality of these products was greatly affected by different environmental conditions and pathogen infection resulting in loss of yield and alteration of the

bioactive content of plants (Cui et al., 2010b). Furthermore, Conceição et al. (2006) stated that field grown plants need a long period to undergo maturity and unfortunately, the cultivars that selected for enhanced secondary metabolite accumulation are not necessarily disease-resistant.

Conventional farming in addition requires hectares of planting area to meet the purpose of providing a large amount of plants material for extraction. This is restricted by sub-urbanization in recent years which contribute to low acreage of agricultural land. Furthermore, many of the plant species used for the isolation of secondary metabolites have to be collected from the wild (Kaiser, 2008; Silva et al., 2013). This will result in great decrease of plant resources due to human intervention in the natural environment (Sarin et al., 2005).

2.2.3 *In vitro* derived source of secondary metabolite

Plant tissue culture technology is an alternative solution in providing plant materials for secondary metabolites harvesting and offers many advantages to the traditional mode of plantation. Secondary metabolites in plant cell cultures can be produced continuously over the year with no seasonal constraints. Besides the advantage of independent ambient weather, the production is also reliable and predictable (Karuppusamy, 2009). This system provides a commercially realistic alternative to whole plant system for secondary metabolite production (Wink et al., 2005). Jeong et al. (2009) stated that cell or organ cultures have appeared as a valuable route for biosynthesizing phytochemicals within a limited time and space. Sarin (2005) highlighted on cultured cells producing a higher level of bioactive compounds than those accumulated in native plants such as shikonin from *Lithorspermum erythrorhizon* and dioesgenin from *Dioscorea* sp. Oksman-Caldentey

and Inze (2004) also reported on low concentrations (0.0005%) of alkaloids, vincristine and vinblastine in the intact plant and hence the use of plant cell culture to produce a higher amount of these alkaloids.

The advantages of cell culture system over the conventional whole plant cultivation has been emphasized by Vijaya Sree et al. (2010) which include: (1) Production of useful bioactive compounds can be done under controlled conditions without depending on external planting factors such as climatic changes or soil conditions; (2) The cultivated cells would be free of microbes and insects; (3) The multiplication of any plant cells, tropical or temperate, could easily be accomplished to yield their specific metabolites; (4) An automation control of cell growth and rational regulation of metabolite processes would reduce labor costs and improve productivity; and (5) Extraction of organic substances are feasible from any type of plant cultures.

2.2.3.1 Callus culture

Callus is an unorganized structure developed on nutrient medium and has the ability to produce secondary metabolites and mass propagation of plantlets. Callus could be established from any type of explants and it is the source material for cell suspension cultures. Over the years, the establishment of callus had been obtained from many plant species. Among successful works on secondary metabolite production from callus cultures in several medicinal plant species are summarized in Table 2.3. However, callus cultures encounter limitations as a source of secondary metabolite production mainly due to slow growth. Hence, cell suspension cultures are prefered for secondary metabolite production (Bourgourd et al., 2001).

Table 2.3: Callus culture as a source of secondary metabolite.

Plant species	Compounds	Reference
Arnebia hispidissima	Alkannin	Shekhawat & Shekhawat, 2011
Artemisia annua	Artemisinin	Baldi & Dixit, 2008
Boesenbergia rotunda	Flavonoids	Yusuf et al., 2013
Buddleja cordata	Phenylpropanoid	Estrada-Zúñiga et al., 2009
Camellia chinensis	Flavones	Nikalaeva et al., 2009
Capsicum annum	Capsiacin	Umamaheswari & Lalitha, 2007
Cassia senna	Sennosides	Shrivastav et al., 2006
Centella asiatica	Asiaticoside	Kiong et al., 2005
Corydylis terminalis	Corydalin	Taha et al., 2008
Coscinium fenustratum	Berberine	Khan et al., 2008
Echinacea angustifolia	Caffeic acid derivatives	Lucchesini et al., 2009
Eriobotrya japonica	Triterpenes	Taniguchi et al., 2002
Frangula alnus	Antraquinones	Kovacevic & Grabisic, 2005
Gymnema sylvestre	gymnemic acid	Gopi & Vatsala, 2006
Momordica chantia	Flavonoids	Agarwal & Kamal, 2007
Nothapodytes foetida	Camptothecin	Thengane et al., 2003
Pluchea lanceolata	Quercetin	Arya et al., 2008
Plumbago rosea	Plumbagin	Komaraiah et al., 2003
Rauvolfia serpentina	Reserpine	Nurchagani et al., 2008
Rhamnus catharticus	Antraquinones	Kovacevic & Grabisic, 2005
Rhazya stricta	3-Oxo-rhazinilam	Gerasimenko et al., 2001
Salvia fruticosa	Rosmarinic acid	Karam et al., 2003
Saprosma fragrans	Antraquinones	Singh et al., 2006
Silybium marianum	Silymarin	Tumova et al., 2006
Solanum tuberosum L.	Glycoalkaloids	Al-Ashaal, 2010
Stevia rebaudiana	Stevioside	Dheerabapattanna et al., 2008
Viccinium myrtillus	Flavonoids	Hohtola et al., 2005
Vitis vinifera	Resveratrol	Kin & Kunter, 2009
Withania somnifera	Steroidal lactone	Mirjalili et al., 2009
Zataria multiflora	Rosmarinic acid	Mohagheghzadeh et al., 2004

2.2.3.2 Cell suspension culture

Cell suspension cultures are undifferentiated cells propagated in liquid medium with agitation to provide maximum aeration for cell growth. The rapid growth of cell suspension culture makes it a material of choice for metabolite production compared to callus cultures and field grown plants (Dicosmo & Misawa, 1995). Even though there are many reports on production of secondary metabolites from cell suspension culture but industrial scale production is still limited.

Chezem and Clay (2016) highlighted that the major challenge in producing secondary metabolites from cell suspension culture was that the metabolites were typically produced by specialized cells and/or at distinct developmental stages. Therefore, undifferentiated plant cell cultures often lose, partially or entirely, their biosynthetic ability to accumulate secondary compounds and in certain condition, some of the compounds were not even synthesized if the cells remained undifferentiated (Charlwood & Charlwood, 1991). Reports on secondary metabolites production in cell suspension cultures technology from various medicinal species are listed in Table 2.4.

In plant cell suspension cultures, the secondary metabolites are synthesized either by secretion into the surrounding medium or stored intracellularly (Zhang et al., 2002). There are two strategies in obtaining secondary product in *in vitro* culture system namely, one-stage or two-stage culture systems (Chen et al., 2003). For single-stage culture system, both growth and production steps are combined together. Whereas, in a two-stage culture system, the first stage involves growing the cells on a standard growth medium and the second stage involves transferring these cells into a production medium suitable for secondary product synthesis (Baque et al., 2012c).

Table 2.4: Secondary metabolite production in cell suspension cultures technology from various medicinal plant species.

Plant species	Compounds	Reference
Ammi majus	Triterpenoid	Staniszewska et al., 2003
Artemisia absinthium	Flavonoids	Ali et al., 2015
Azadirachta indica	Azadirachtin	Sujanya et al., 2008
Brucea javanica	Cathin	Wagiah et al., 2008
Camptotheca acuminata	Isocamptothecin A and B	Yu et al., 2005
Cassia acutifolia	Anthraquinones	Nazif et al., 2000
Catharanthus roseus	Catharanthine Alkaloid Vincristine	Ramani & Jayabaskaran, 2008; Zhao et al., 2001 Lee-Parsone & Rogce, 2006
Capsicum frutescens	Capsaicin	Sudha & Ravishankar, 2003
Cayratia trifoliata	Stilbenes	Raot & Ramawat, 2009
Centella asiatica	Centellosides and phytosterols	Bonfill et al., 2010
Coscinuim fenustratum	Berberine	Narasimhan & Nair, 2004
Eleutherococcus senticosus	Eleuthrosides	Shohael et al., 2007
Eucommia ulmoides	Chlorogenic acid	Wang et al., 2003
Hssopus officinalis	Sterols	Skrzypek & Wysokinsku, 2003
Hypericum perforatum	Hypericins, hyperforins and flavonoids	Pasqua et al., 2003
Hyoscyamus muticus	Alkaloid	Aly et al., 2010
Larrea divaricata	Phenolic	Palacio et al., 2011
Mentha piperita	Rosmarinic acid	Krzyzanowska et al., 2012
Morinda citrifolia	Anthraquinones	Quevedo et al., 2010
Panax ginseng	Ginsenosides	Jeong et al., 2008
Pueraria tuberosa	Isoflavonoids	Goyal & Ramawat, 2008
Podophyllum hexandrum	Podophyllotoxin	Chattopadhyay et al., 2002
Stizolobium hassjoo	L-Dopa	Sung and Huang, 2000
Taxus baccata	Taxol	Khosroushahi et al., 2006
Taxus chinensis	Paclitaxel, Taxol	Choi et al., 2000; Fan et al., 2006
Taxus cuspidate	Taxol and baccatin III	Fett-Neto et al., 1995
Tinospora cordifolia	Berberine	Rama Rao et al., 2008
Withania somnifera	Withanolide A	Nagella & Murthy, 2010
Zingiber zerumbet	Zerumbone	Jalil et al., 2015

2.2.3.3 Shoot culture

There are a number of efforts to develop shoot cultures aiming for overproduction of pharmaceutically significant compounds such as artesiminin, hypericin, indirubin, bacoside A, phenolics and flavonoids from various plant species (Smetanska et al., 2008). The approaches used in targeting high compound accumulation in shoot cultures include cultural conditions, growth hormone and introduction of elicitor culture medium.

Charchoglyan et al. (2007) stated that optimization of plant growth regulator such as BA and NAA enhanced shoot culture-derived compound that is hyperforin and secohyperforin in *Hypericum perforatum*. Whereas, Praveen et al. (2009a) investigated the effects of plant growth regulators with semisolid and liquid media on bacoside A accumulation and observed the highest saponin (11.9 mg/g dry weight) accumulation in liquid medium. The shoot culture of *Hypericum maculatum* on optimized medium showed approximately two-fold increased in hypericin production compared to controls, and the growth of *H. hirsutum* shoots cultivated on the same medium led to six-fold increase of hyperforin (Coste et al., 2011). Recently, Largia et al. (2015) has successfully enhanced bacoside A content in shoot cultures of *Bacopa monnieri* through elicitation. Table 2.5 shows the secondary metabolite production from shoot cultures of various plants.

Table 2.5: Shoot cultures for secondary metabolite production.

Plant species	Compounds	Reference
Artemisia annua	Artemisinin	Paniego & Giulietti, 1996
Adhatoda vasica	Vasine	Shalaka & Sandhya, 2009
Ammi majus	Umbelliferone	Krolicka et al., 2006
Bacopa monnieri	Bacoside A	Largia et al., 2015
Buddleja cordata	Verbacoside and phenols	Estrada-Zūñiga et al., 2016
Centella asiatica	Asiaticoside	Kim et al., 2004b
Cymbopogon citratus	Essential oils	Quiala et al., 2006
Drosera rotundifolia	7-methyljuglone	Hohtola et al., 2005
Echinacea angustifolia	Alkamides	Lucchesini et al., 2009
Fritillaria unibracteata	Alkaloids	Gao et al., 2004
Gentianella austriaca	Xanthone	Vinterhalter et al., 2008
Harpagophytum procumbens	Phenolic and gallotannin	Bairu et al., 2010
Hemisdesmus indicus	Lupeol, rutin	Misra et al., 2005
Hypericum maculatum	Hypericins and hyperforin	Coste et al., 2011
Hypericum perforatum	Hypericins	Kornfeld et al., 2007
Hypericum perforatum	Hyperforin	Karppinen et al., 2007
Hypericum perforatum	Hypericin and pseudohypericin	Kirakosyan et al., 2000
Hypericum perforatum	Hypericins derivates	Zdunek & Alfermann, 1992
Hypericum perforatum	Naphtodianthrones and phenylpropanoids	Gadzovska et al., 2013
Hypericum perforatum	Hypericins, hyperforins and flavonoids	Pasqua et al., 2003
Hypericum rumeliacum	Phenolics and flavonoid	Danova et al., 2010
Polygonum tinctorium	Indirubin	Shim et al., 1998
Psoralea corylifolia	Isoflavones	Shinde et al., 2009
Salvia officinalis	Flavonoids	Grzegorczyk & Wysokinska, 2008

2.2.3.4 Hairy root culture

Hairy root culture is one of the alternatives to agricultural practices for producing valuable secondary metabolites. Transgenic hairy root cultures have enhanced the role of plant tissue culture in secondary metabolite production. Choi et al. (2000) described hairy root as fine adventitious root grown from explants which had been transformed by *Agrobacterium rhizogenes* plasmid. Their distinctive characters are based on their genetic and biosynthetic stability, more rapidly in growth, and easily maintained (Ramachandra & Ravishankar, 2002). By using this methodology, a broad range of chemical compounds have been produced (Giri & Narasu, 2000). *Agrobacterium rhizogenes* infected root cultures provided productivity which exceeded the callus of normal roots. Furthermore, the ability of these hairy roots to undergo rapid propagation without an external supply of auxins remained attractive for metabolite production. Due to these advantages, several of the root derived plant metabolites for production by *in vitro* culture were considered for production using the hairy root cultures system. Some studies of the production of secondary metabolites from hairy roots of medicinal plants are listed in Table 2.6.

Table 2.6: Hairy root cultures producing pharmaceutical products of interest.

Plant species	Compounds	Reference
Agastache rugosa	Rosmarinic acid	Lee et al., 2007a
Ammi majus	Furanocoumarins	Królicka et al., 2001
Angekica gigas	Deoursin	Xu et al., 2008
Arachys hypogaea	Resveratol	Kim et al., 2008
Atropa belladona	Tropane alkaloid	Yang et al., 2011
Azadirachta indica	Azadirachtin	Satdive et al., 2007
Brugmansia candida	Tropane alkaloid	Marconi et al., 2008
Catharanthus roseus	Alkaloid	Gaviraj & Veeresham, 2006
Catharanthus roseus	Ajmalicine	Thakore et al., 2015
Centella asiatica	Asiaticoside	Kim et al., 2007
Datura metel	Tropane alkaloids	Cusidó et al., 1999
Fagopyrum esculentum	Rutin	Lee et al., 2007b
Glycyrrhiza glabra	glycyrrhizin	Mehrottra et al., 2008
Hyoscyamus niger	Tropane alkaloids	Zhang et al., 2011
Nicotiana tabacum	Nicotine	Zhao et al., 2013
Panax ginseng	Ginsenoside	Yu et al., 2005a; Zhang et al., 2015
Panax ginseng	Glycoside	Jeong & Park, 2007
Physalis minima	Physalins	Azlan et al., 2002
Plumbago zeylanica	Plumbagin	Verma et al., 2002
Rauvolfia micrantha	Ajmalicine	Sudha et al., 2003
Rubia akane	Antraquinone	Park & Lee, 2009
Rubia tinctorum	Antraquinone	Sato et al., 1991
Silybum marianum	Silymarin	Hasanloo et al., 2013
Salvia miltiorrhiza	Diterpenoid tanshiones	Yan et al., 2005
Solanum chrysotrichum	Saponin	Caspeta et al., 2005
Tagetes patula	Thiophene	Mukundun & Hjortsø, 1991
Taxus x media	Taxane	Syklowska-Baranek et al., 2015
Withania somnifera	Withanoloid A	Murthy et al., 2008

2.2.3.5 Adventitious root culture

Unlike undifferentiated cells in cell suspension cultures which may lack the biosynthetic machinery required for the generation of secondary metabolite of interest, adventitious roots can be mass propagated in liquid media as differentiated tissue. In terms of plant organ culture, root culture is more favourable than other culture system due to its rapid multiplication and the ability to be scaled up in bioreactor (Saiman et al., 2012). Adventitious roots grow vigorously in phytohormone-supplemented medium showing high rate of proliferation and great potential for valuable secondary metabolites accumulation (Hahn et al., 2003; Yu et al., 2005; Murthy et al., 2008).

Roots are the site for biosynthesis or accumulation of major secondary metabolites, including alkaloids, napthoquinones, polyacetylene and sesquiterpenes (Thwe et al., 2012). Murthy et al. (2008) highlighted the four discrete stages in secondary metabolite production from adventitious root, namely: (1) Induction of adventitious roots from the explants of choice (callus mediated or direct induction); (2) Cultivation of adventitious roots in liquid medium, in flask-scale cultures and establishing growth kinetics (developing suitable medium components and cultural environment for the biomass and metabolite accumulation), (3) Development of strategies for higher metabolites accumulation (medium selection, elicitation strategy or precursor feeding), (4) Large scale cultivation of adventitious roots in using bioreactors (developing suitable methodology for up-scaling cultivation). Table 2.7 shows adventitious root cultures of medicinal plants producing bioactive secondary metabolites.

Table 2.7: Adventitious root cultures for secondary metabolite production.

Plant species	Compounds	Reference
Aleo vera	Aloe-emodin	Lee et al., 2011
Artemisia annua	Artemisinin	Paniego & Giulietti, 1996
Andrographis paniculata	Andrographolide	Praveen et al., 2009b
Astragalus membranaceus	Astragaloside, flavones	Thwe et al., 2012
Cornus capitata	Irridoids	Tanaka et al., 2001
Castilleja tenuiflora	Phenylethanoid glycosides	Gómez-Aguirre et al., 2012
Echinacea angustifolia	Phenolics	Wu et al., 2006
Echinacea purpurea	Caffeic acid derivatives	Wu et al., 2008, Jeong et al., 2009
Eleutherococcus koreanum	Eleuthrosides B & E, chlorogenic acid phenolic, flavonoid	Lee et al., 2015
Eurycoma longifolia	Phenolic, flavonoid	Lulu et al., 2015
Glycyrrhiza uralensis	Glycyrrhizic acid, glycyrrhetinic acid, polysaccharide	Li et al., 2016
Hypericum perforatum	Chlorogenic acid, hypericins, hyperforins and flavonoids	Pasqua et al., 2003; Cui et al., 2010a
Morinda citrifolia	Anthraquinone, phenolics, flavonoids	Baque et al., 2012a
Panax ginseng	Ginsenosides, linoleic and α-linolenic, saponin	Kim et al., 2005; Wu et al., 2008; Wu et al., 2009; Paek et al., 2009
Periploca sepium	Periplocin	Zhang et al., 2011
Podophyllum peltatum	Podophyllotoxin	Anbazhagan et al., 2008
Polygonum tinctorium	Indigo, indirubin	Shim et al., 1998
Prunella vulgaris	Flavonoids	Fazal et al., 2014
Psammosilene tunicoides	Saponin	Zhang et al., 2013
Pseudostellaria heterophylla	Saponin, polysaccharide	Yin et al., 2013
Raphanus sativus	Anthocyanin	Betsui et al., 2004
Rhus javanica	Galloylglucose, reccionidin	Taniguchi et al., 2000
Salvia fruticosa	Rosmarinic acid	Karam et al., 2003
Scopolia parviflora	Tropane alkaloid	Min et al., 2007
Withania somnifera	Withanolide	Sivanandhan et al., 2012

2.3 PLANT CELL CULTURE ENHANCEMENT STRATEGIES

Current advances in plant biotechnology provide an opportunity to produce useful secondary metabolites through plant cell, tissue or organ cultures instead of whole plant cultivation. However, large scale production of valuable secondary metabolites by plant cell culture for commercial purposes has been known to be impractical due to its poor productivity and instability. Furthermore, some of the useful compounds are not synthesized in the undifferentiated cells (Kim et al., 2002; Rao & Ravishankar, 2002; Verpoorte et al., 2002). There are many reports on low accumulation of secondary compounds in tissue culture system such as callus and cell suspension cultures. Therefore, yield from the cell culture need to be increased in order to make it commercially viable (Alfermann & Petersen, 1995).

In optimized culture conditions, several compounds in plant cell culture systems could produce higher yield than field-grown plants. Other than cultural conditions optimization, efforts have been made to achieve the desired yield by selecting high producing strains and employing precursor feeding, transformation methods, and immobilization techniques (Karuppusamy, 2009).

2.3.1 Strain improvement

Cell line selection is one of the conventional and effective approaches in enhancing metabolites accumulation in cell cultures. One of the critical steps to obtain high yielding cell lines is the selection of parent plant. Generally, high-producing plants give rise to high-producing cell lines, even though the production levels in plant cells are variable. High-producing cell lines with suitable genetics, biochemical and physiological characteristics, and medium optimizations could lead to enhancement in secondary metabolite production (Zhong, 2001; Karuppusamy, 2009).

However, variability in cell lines often leads to reduction in productivity with regular subculturing and has been attributed to genetic changes in the culture, or epigenetic changes, which were due to physiological conditions (Smulders & de Klerk, 2011; Landey et al., 2015). Non-uniform cell physiological characteristics could result in a decrease of metabolites productivity of the cell culture. To overcome these problems, separation of elite clones during cultivation phase and reverse changes of culture environment were required (Dörnenburg & Knorr, 1995).

2.3.2 Media optimization

Plant cell culture medium contains phytohormones, organic and inorganic components. The effect of medium used on various processes has been reported in many studies on secondary metabolites accumulation (Zhong, 2001). Medium with different composition affects growth of cell cultures as well as metabolite production. Lopez et al. (2016) stated that adjustment of the basal culture medium for *in vitro* plant cultures is needed to ensure high production of secondary metabolites. The selection of basal medium was found to influence the production of secondary metabolites with antibacterial and antioxidant properties from *Clidemia hirta* cell cultures (Lopez et al., 2016). In another case, the production of ginsenosides was affected by different concentrations of carbon, nitrogen, phosphate and plant growth hormones (Kochan *et al*., 2016). Suitable nutrient strength was not only crucial to produce good growth of cells, but also important for secondary metabolite production (Ramachandra & Ravishankar, 2002) which have been hypothesized to be linked to the ionic balance in the medium (Drewes & Staden, 1995). Lee and Paek (2012a) reported that by increasing medium strength, production of eleutheroside B and E, chlorogenic acid, total phenolics, and flavonoids in contrast, showed a decline pattern.

Plant cell cultures are normally cultivated by using simple sugars as a carbon source. Carbon source was found to be an important factor in plant metabolism and was among the extensively studied parameter for biomass optimization and compound harvesting. Most *in vitro* cultures are autotrophic-incompetent and unable to proliferate well without exogenous supply of carbohydrates (Cournac et al., 1991). Narayan and Venkataraman (2002) affirmed that carbon source affect the production of anthocyanin in carrot cell cultures. The carbon source has also effected the accumulation of anthocyanins in *Vitis vinifera* cell suspensions (Do & Cormier, 1991) and shikonin in *Lithospermum erythrorhizon* cell cultures (Srinivasan & Ryu, 1993). Ketchum et al. (2003) also reported that an increased production rate of paclitaxel in the culture medium was achieved prior carbohydrate addition during the growth cycle.

Generally the best carbon sources for most plant cell cultures are sucrose, glucose and fructose. In various studies, it has been ascertained that among these carbon sources, sucrose was the most favourable and provided advantages for the cultivation of cell cultures from various plant species (Wu & Zhong, 1999). In suspension culture of ginseng cells (*Panax* spp.), sucrose manipulation was confirmed to be incredibly effectual in culture productivity improvement as well as production of ginseng saponin and polysaccharide (Zhang et al., 1996). As for *Z. zerumbet* cell suspension cultures, the production of zerumbone was significantly affected by different carbohydrate substrate and sucrose was found to be the best carbohydrate substrate for biomass accumulation and zerumbone production (Jalil et al., 2015). Sucrose was replaced by other carbohydrate substrate to enhance secondary metabolite production. Kim et al. (1995) determined the production of paclitaxel from *Taxus brevifolia* cell suspension cultures in optimized medium containing 6% fructose after 10 days of cultivation. Similarly, the production of catharantine was doubled in *Catharanthus roseus* cultures when fructose

was used as a carbon source (Kim, 2001).

It was also reported that the concentration of sucrose affected the biomass growth and secondary metabolite production. Increased sucrose concentration typically resulted in increased biomass and metabolite production of plant cell cultures. This was proven in suspension cultures of *Anchusa officinalis* by using a perfusion medium where sucrose concentration which was two-fold higher than normal growth medium resulted in an increase of the final cell density and the rosmarinic acid content by more than 2-fold compared with a batch culture without medium exchange (Su & Humphrey, 1990). The increasing metabolites accumulation due to high sucrose concentration was also observed in *Catharanthus roseus* callus culture (Zhao et al., 2001a). In contrast, for *Hypericum perforatum*, high sucrose concentration (5, 7 and 9%) inhibited the biomass accumulation of root culture but enhanced the amount of total phenols, flavonoids, chlorogenic acids and total hypericin content (Cui et al., 2010b). The sucrose concentration used for the crocin synthesis was rather low, 30 g/L in comparison to higher concentration (45 g/L) that support saffron callus growth (Liu et al., 2002). This concluded that carbohydrate types and concentrations play a major role in biomass accumulation and compound production.

Phytohormones have displayed the most significant effects on cell growth and plant metabolites productivity (Aly et al., 2010). Auxin and cytokinin profoundly influence growth as well as product formation in plant cell cultures. Studies based on the type and concentration of phytohormones and their single or synegistic effect were commonly carried out. Zhong et al. (1996) stated that plant growth regulators concentration in the medium influenced the cell growth and ginsenoside production in suspension cultures of *Panax quinquefolium*. Auxin stimulated phenolic compounds and flavonoids synthesis

in *Cuphea aequipetala* (Martínez-Bonfil et al., 2014) and the production of camptothecin in *Ophiorrhiza prostrate* adventitious root cultures (Martin et al., 2008). Eventhough, excessive level of auxin was claimed to be good for cell growth but it could adversely affect the secondary metabolite production. This was reported by Narayan et al. (2005) who observed the suppression of anthocyanin production in carrot cell cultures. Auxin in combination of cytokinin was also shown to inhibit the synthesis of myricetin and naringenin in *Rumex crispus* adventitious root cultures (Mahdieh et al., 2015). The addition of gibberelin, also sometimes provide positive effect on secondary metabolite production in cell culture. This was proven by a study by DiCosmo and Misawa (1995) that stated the addition of gibberellic acid into cell culture encourage the growth of callus growth and taxol production in *Taxus cuspidata.*

Stafford (1991) stated that high biomass production rates have adverse effects on the secondary metabolites production. In a study performed on shoot cultures of sage, under the influence of plant growth regulators (cytokinins, 2,4-D and BA), it was found to increase the rates of shoot proliferation with linear shoot growth. In contrary, it resulted in reduced antioxidant phenolics production (Santos-Gomes et al., 2002). In contrast, the combination of auxin and cytokinin resulted in a significant reduction of *Morinda citrifolia* root biomass, but increased in the secondary metabolite production (Baque et al., 2010a).

Nitrogen source are also vital for plant cell metabolites formation. Nitrogen served as another main component that significantly affects the cell growth and metabolite formation (Cui et al. 2010c; Lee & Paek 2012b; Zhang et al., 2011). Investigation by Cui *et al*. (2010c) discovered that medium supplemented with ammonium/nitrate ratio (NH_4^+/NO_3^-) of 5:25 (mM) resulted in the optimum biomass accumulation as well as

total phenols and flavonoids in *Hypericum perforatum* adventitious root cultures. While supplementation of an equal ammonium/nitrate ratio (NH_4^+/NO_3^-) of 30:30 provided the optimum saponin production in adventitious roots cultures of *Pseudistellaria heterophylla* (Yin et al., 2013). In *Salvia fructicosa* cell cultures, the addition of phenylalanine which resulted in excess nitrogen increased the rosmarinic aid accumulation (Karam et al., 2003).

2.3.3 Culture conditions

Varying culture condition is another strategy in enhancing secondary metabolite production in plant cell cultures. Optimized physical and chemical factors such as light intensity, inoculum density, temperature, agitation and pH have influenced the accumulation of targeted secondary metabolites in cell cultures.

The influence of light on secondary metabolite production varied between species and is one of the major factors being investigated for many years. The spectral quality, intensity and period of light irradiation have affected plant cell culture in one aspect or another (Zhong et al., 1991). Topchiy et al. (2005) also reported that the differences in spectral radiation quality influenced the composition of proteins, which is an important characteristic for the functional state of the cell metabolic activity. Fett-Neto et al. (1995) highlighted on the importance of light and nutrients optimization of paclitaxel production in *Taxus suspidata* cell cultures. Heo et al. (2002) also mentioned that light controlled growth and differentiation of plant cell, tissue and organ cultures. The effect of light on biomass accumulation and compound production varied between species. In some species, light positively enhanced biomass and compound production. For example, in *Morinda citrifolia* adventitious root culture, light quality significantly affected the biomass and the total anthraquinone, phenolic, and flavonoid contents

(Baque et al., 2010a). In other species the effect of light on biomass accumulation and metabolites production was contrasting where light condition event hough promoted better growth but suppressed the alkaloid content in *Hyoscyamus muticus* cell suspension cultures (Aly et al., 2010).

Inoculum density is another essential factor which affects the biological environment of cultures due to cell to cell and cell to medium interaction (Aly et al., 2010). Recent studies in several plant cell cultures showed that secondary metabolite yields were sturdily dependent on the size or density of the inoculum when cultures were initiated (Nguyen et al., 2012; Pillai et al., 2015). Lee and Shuler (2000) reported that high inoculum density of *Catharanthus roseus* cells cultures produced high ajmalicine concentrations comparatively. Later Zhang et al. (2002a) stated that the intracellular paclitaxel production was enhanced in *Taxus yunnanensis* cell suspension cultures when a moderately high inoculum (200 g FW) size was used. Recent study by Yusof et al. (2013) showed that low initial inoculation volume (0.25 ml SCV) compared to (optimal inoculation) resulted in poor biomass of cells suspension of *B. rotunda* and low flavonoids yield which perhaps due to inadequate critical mass of surviving cells and degree of nutrient abundance in the liquid media which lead to negative effect on the cell growth. Nguyen et al. (2012) also stated that low initial inoculum (2% w/v) showed highest growth rate in adventitious root of *Panax vietnamensis*. Zhang et al. (2002a) mentioned that for *Taxus yunnanensis* cell cultures at most inoculum ages, the inoculum size change had minute or no significant effect on the specific taxol yield. Therefore, the effect of inoculum size on volumetric taxol yield and productivity was primarily a result of its effect on biomass production rather than taxol biosynthesis.

Temperature ranging from 17 to 25°C is generally employed for callus induction and growth of plant cell cultures but each plant species may favour a different temperature. In *Z. zerumbet* cells suspension culture, low temperature (18°C) negatively affects the cell growth and zerumbone production (Jalil et al., 2015). Previous study stated that lowering the cultivation temperature resulted in an increased of total fatty acid content per cell in dry weight for *Catharanthus roseus* cells suspension culture (Toivonen et al., 1992). Further investigation reveals that the optimum temperature for plants cell growth was at 24°C while paclitaxel synthesis was produced at a maximum rate at 29°C (Choi et al., 2000a). These conclude that each plant species has different optimized temperature both for cells growth and bioactive compounds production. Therefore, it is necessary to optimize the nutrient composition and culture condition in each of the species studied.

2.3.4 Elicitors

Elicitors refer to chemicals that can trigger physiological and morphological responses and metabolite accumulation (Zhao et al., 2005). This includes abiotic elicitors such as metal ions and inorganic compounds, while biotic elicitors ranging from bacteria, viruses, fungi or herbivores as well as plant cell wall components. The treatment with biotic and abiotic elicitors is a simple and efficient approach for the stimulation of secondary metabolite production in plant cell cultures. The use of elicitors activates plant defense mechanisms which resulted in significant production of bioactive secondary metabolites (Jeandet et al., 2016). Khouroushahi et al. (2006) stated that application by using elicitors increased taxol production up to 16-fold higher than that of untreated control in *Taxus baccata* cell suspension culltures. Previously, a range of biotic and abiotic elicitors have been extensively used to enhance the production of taxol in *Taxus* spp. cultures, including fungal components (Zhang et al.,

2000; Yuan et al., 2002), benzoic acid (Fett-Neto et al., 1994), methyl jasmonate (Linden & Phisalaphong, 2000), arachidonic acid (Namdeo, 2007), silver ion (Zhang et al., 2000) and chitosan (Linden & Phisalaphong, 2000; Zhang et al., 2000).

Among the commonly used biotic elicitors are jasmonate derivative, methyl jasmonate (MeJA), salicylic acid, oxalic acid, putrescine and chitosan. Chitosan was found to increase the production of stilbene in *Vitis vinifera* cells cultures (Ferri et al., 2011). MeJA elicitation increased eleutherosides and chlorogenic acid production in embryogenic suspension cultures of *Eleutherococcus sessiliflorus* (Shohael et al., 2008). The addition of MeJA or dihydromethyl jasmonate to suspension cultures increases the production of ginsenosides (Wang & Zhong, 2002). In *Podophyllum peltatum*, methyl jasmonate (20 μM) strongly enhanced podophyllotoxin production in both embryogenic cells and adventitious roots cultures (Anbazhagan et al., 2008). High yield of secondary metabolites were detected for *Taxus chinensis* and *Panax ginseng* cell cultures by adding the MeJA and jasmonate derivatives (Thanh et al., 2005). A significant increase in accumulation of hypericin was observed in the cell cultures of *Hypericum perforatum* subsequent to the addition of jasmonic acid (Walker et al., 2002). Gadzovska *et al*. (2013) observed that salicylic acid induced double production both hypericin and pseudohypericin in cell suspension cultures but not in callus and shoot culture of *H. perforatum*.Synegistic effect of elicitors on compounds accumulation was also reported. Recently, Largia et al. (2015) reported on the enhancement of bacoside A in shoot cultures of *Bacopa monnieri* through synegistic effect of methyl jasmonate and salicylic acid.

The usage of abiotic elicitor, such as vanadate increased ginsenoside accumulation in *Panax ginseng* cell culture (Huang & Zhong, 2013). Georgiev et al. (2005) also pointed out that elicitation with vanadyl sulfate enhanced rosmarinic acid production in *Lavandula vera* MM cell suspension cultures. Similarly, Zhang et al. (2013) affirms the addition of exogenous oxalic acid in the liquid culture of *Psammosilene tunicoides* saponins adventitious roots producing higher accumulation of total saponins than in control culture. For organic elicitor, Hara et al. (1991) has reported on the influence of spermidine in berberine production of *Thalictrum minus* cell cultures. Sudha and Ravishankar (2003) reported that putrescine supplementation to cell suspension cultures of *Capsicum frutescens* was found to be effective in enhancing both biomass and secondary metabolite production. However, the response varied and depended upon cell lines, concentrations of elicitor and the stage of the growth phase at which the cells were treated.

For fungal elicitor, Namdeo et al. (2002) reported that ajmalicine accumulation in *Catharanthus roseus* cell suspension cultures was increased by about 3-fold when cells were treated fungal cell wall fragment of *Aspergillus niger, Fusarium moniliforme*, and *Trichoderma viride*. Sarin (2005) also observed the increasing yields by using yeast polysaccharide elicitor but obtained relatively low *T. rugosum* biomass Other elicitor including lanthanum (trivalent ion of a rare earth element) which was tested for elicitor-like effects on the production of taxol in cell suspension cultures of four different *Taxus* spp. The maximum accumulation of taxol was attained by the addition of La^{3+} (5.8 μM) to the culture during mid-log growth phase, increasing the volumetric taxol yield by nearly three-folds (Wu et al., 2001).

The addition of elicitors to plant cell cultures may hinder the accumulation of biomass since elicitation typically switches primary metabolism to secondary metabolism in the cells. Such an adverse effect has commonly been observed in *Taxus* cell cultures (Zhang et al., 2000). Therefore, a two-stage culture strategy was adopted in order to improve taxol production in *Taxus* cell suspension cultures. In the first stage, the medium and cultural conditions were optimized for maximum biomass growth, while in the second stage; the culture was maintained in a medium supporting taxol formation and later treated with selected elicitors to stimulate high taxol synthesis (Zhang et al., 2000).

2.3.5 Precursors

Besides elicitation, precursor feeding is another strategy for enhancing the metabolites production in plant cell cultures. There are many reports on secondary metabolites enhancement by the addition of suitable precursors or related compounds into the culture media. For example, the feeding of geraniol, 10-hydroxygeraniol, or loganin at 21 days each in *Catharanthus roseus* resulted in significant increases in the accumulation of tabersonine (Morgan & Shanks, 2000). Woerdenberg et al. (1990) applied a combination of precursors, conifery1 alcohol, and b-cyclodextrin to increase the yield of podophyllotxin in *Podophyllum hexandrum* cell suspension cultures.

Similarly, the addition of 3 mM conifery1 alcohol complex to *Linum flavum* yielded 0.013% podophyllotoxin on a dry weight basis, whereas cultures without the precursor only produced up to 0.0035% compound (Koulman et al., 2003). Gaviraj and Veeresham (2006) reported on multiple feedings of loganin increased the accumulation of alkaloids in *Catharantus roseus* cell culture, supporting the importance of terpenoid pathway in alkaloid biosynthesis. Production of numerous bioactive compounds such

as tropane alkaloids, indole alkaloids, and phenolic compounds were also enhanced by the supplementation of appropriate amino acid. Nevertheless, the addition of precursor sometimes enhanced the biomass growth but suppressed compound production. This was proven by Palacio et al. (2011), who reported that the addition of cinnamic acid enhanced the growth of *Larrea divaricata* cell culture, but not the phenolic compounds production.

2.3.6 Metabolic engineering

Metabolic engineering is a highly potential approach in producing important secondary metabolites. To enhance the productivity of commercially important bioactive compounds in plants or plant cell cultures, or even to produce completely new compounds, Verpoorte et al. (1999) emphasized that metabolic engineering of plant secondary metabolite pathways has opened up another new perspective. Metabolic engineering of cells is a natural outcome of recombinant DNA technology. A remarkable finding by Muir et al. (2001), which have successfully upregulated flavonol biosynthesis in tomato in order to increased antioxidant capacity involved transformation using *Petunia* chi-a gene encoding chalcone isomerase. In a wide-ranging sense, metabolic engineering can be viewed as the biochemical reaction networks design to accomplish a certain objective. In general, the aim is either to enhance the rate of a desired product or to reduce the rate of undesired side-products, or to decompose the toxic or undesired substances (Zhong, 2001).

The demand of using natural products for medicinal purposes is increasing, and metabolic engineering can alter the pharmaceuticals production and assist to design new therapies. At the present time, scientists aspire to produce substances with antitumor, antiviral, hypoglycaemic, anti-inflammatory, antiparasitic, antimicrobial, tranquilizer

and immuno-modulating activities through tissue culture technology (Vijaya Sree et al., 2010). For instance, since chemically synthesized artemisinin is not commercially viable presently, metabolic engineering was adopted to acquire a higher content of artemisinin in transgenic plants. This provides a promising approach to reduce the price of artemisinin-based antimalarial drugs in the market (Liu et al., 2011). Transcription factors are also used as molecular tools for plant metabolic engineering to boost the production of valuable bioactive compounds (Gantet & Memelink, 2002).

Even though engineering of plant secondary metabolism is feasible nowadays, however it necessitates knowledge of the biosynthetic pathways involved. In order to increase the desired compounds, numerous metabolic pathways in that particular plant need to be studied and well understood. Metabolic engineering also require a specific knowledge on the enzymes related to the pathways, enzyme characterization, regulation and activity. To enhance secondary metabolite production, different strategies can be pursued, such as overcoming rate-limiting steps, reducing flux through competitive pathways, reducing catabolism and overexpression of regulatory genes (Verpoorte et al., 2000). For example, gene cloning can be targeted to enhance the metabolic flux in order to improve the productivity of the targeted metabolite(s) of the plant or plant cell culture system (Bhatia, 2015). This purpose can be accomplished either by overexpressing the target pathway or suppressing other pathways.

Study by Quevedo (2010) revealed higher total phenolics and phenylalanine ammonia-lyase activity in the transgenic line of *Morinda citrifolia* cell line obtained by overexpression of 1-deoxy-D-xylulose 5-phosphate synthase (DXS) from *Catharanthus roseus*, a key enzyme of the metabolic pathway of anthraquinones. Most recently, Zhang et al. (2015) reported on overexpression of α-L-rhamnosidase gene into hairy

root culture which resulted in 2.2-fold higher ginsenoside Rg_1 in *Panax ginseng*. Forkmann and Marten (2001) also highlighted on other strategies by introducing novel structural or regulatory genes, and the antisense or sense suppression of genes in a particular pathway. Introduction of two *O*-methyltransferase [*Coptis japonica* norcoclaurine 6-*O*-methyltransferase (6OMT) and 3′-hydroxy-*N*-methyl coclaurine 4′-*O*-methyltransferase (4′OMT)] expression vectors into California poppy cell cultures in order to prevent gene silencing effect of endogenous genes showed improve benzylisoquinoline alkaloids production in *Coptis japonica* (Inui et al., 2006).

2.4 BIOREACTOR

A bioreactor is a vessel in which a biological reaction or change occur involving biological systems such as enzymes, microorganisms, plant or animal cells, and tissues (Zhong, 2010). Paek et al. (2005) described bioreactor as self-contained sterile environments which designed for intensive culture and exploit on liquid nutrient to afford maximal opportunity for monitoring and control over micro environmental conditions for instance aeration, agitation, dissolved oxygen, temperature and pH. Various types and configurations of bioreactor have thus been exploited and developed along with the advances in the understanding of biological systems. The bioreactor types including spin filter, continuously strirred turbine, hollow fibre, two-step immobilization, strirred tank, air-lift, rotating drum, and photo (Zhong et al., 1995). An efficient bioreactor system depends greatly on its control and support systems, which closely and efficiently integrated into the whole production system. In order to support the desired functions of the living cells or enzymes, it is necessary to control and optimise the bioreactor operating variables such as dissolved oxygen concentration, pH, temperature, mixing, and supplementation of nutrients (Zhong, 2010).

Bioreactors perform as a biological factory for the production of high-quality products which offer many advantages. Among the advantages are controlled supply independent of plant availability, homogeneous culture caused by mechanical or pneumatic stirring mechanism, better control of cultural and physical environment, reproducible yields of end product, enhanced nutrient uptake stimulating multiplication and the opportunity to perform biosynthetic and/or biotransformation experiments related to metabolite production with enzyme availability (Su, 2006).

2.4.1 Plant bioreactor

Optimization of the plant cell culture conditions in bioreactor system is crucial with respect to the physical parameters such as mixing and shear stress. Glass and stainless steel bioreactors incorporating impellers are often used for cell and/or tissue culture production at industrial scale (Yesil-Celiktas et al., 2010). The understanding on how to promote better cell growth through reactor modification, such as the use of appropriate impeller designs that generate not as much of shear is a major challenge (Srivastava & Srivastava, 2012). Another important factor to be considered is the implementation of aeration technologies that constantly need to be improved (Yesil-Celiktas et al., 2010). So, it is of major importance to design bioreactor configurations, which can offer sufficient mixing and mass transfer while minimizing the intensity of shear stress and hydrodynamic pressure. Nevertheless, recent studies by Srivastava and Srivastava (2007) on the shear sensitivity of plant cells showed that actually plant cells in general are quite shear-stress tolerant. This has been proven by large-scale propagation of shikonin from plant cell cultures in a $60m^3$ stirred tanks (Min et al., 2007). Another approach by Georgiev et al. (2011) is by using a pulse-aerated column reactor designed for high production of verbascoside, one of the highest yield reported by suspension cultures of *Harpagophytum procumbens.*

2.4.2 Plant bioreactor application

2.4.2.1 Micropropagation

Bioreactor is mainly used in production of cells biomass that includes organogenic product such as shoots or roots, or embryogenic cells. Biorector is also widely applied in producing metabolites and enzymes and for biotransformation of exogenously added metabolites through the usage of precursors in a metabolic pathway (Paek et al., 2005). Automation of micropropagation in bioreactors has been reported by several scientists as a possible way of reducing production costs (Preil, 1991; Sharma, 1992; Takayama & Akita, 1994; Christie et al., 1995; Leathers et al., 1995; Son et al., 1999; Ibaraki & Kurata, 2001; Chakrabarty & Paek, 2001; Paek et al., 2001). Since then it has proven to be applicable to many species and plant organs including shoots, bulbs, microtubers, corms and somatic embryos (Paek et al., 2001).

2.4.2.2 Biomass and secondary compound production

The optimization of bioreactor configurations and environmental condition are crucial for large scale production of useful plant cells metabolites for feasibility of commercialization (Zhong, 2001). The cultivation of plant cell or root cultures can now be feasible in small scale bioreactors with various configurations. However, for large to commercial scale production, various unique properties of bioreactors for plant cell or root cultures such as fluid mixing, shear sensitivity, low oxygen requirements and slow growth rates are manifested in complex ways (Baque et al., 2012b). With the increase in up-scaling process, mixing inside the reactor becomes complicated; resulting in non-uniform nutrients concentration and limited oxygen transfer to respiring cells or roots. Furthermore, the clumping and cells wall growth inside the reactor vessel caused by modified rheological nature of the fluid, give rise to sedimentation that significantly affects cell growth and product formation during bioreactor cultivation (Chattopadhyay

et al., 2002; Zhong, 2010). Among hundreds of plant secondary metabolites that have been produced through cell cultures; only shikonin, ginsenosides and berberine are presently produced on a large scale and indeed these are the most successful stories of an industrial scale-up linking plant cell culture with bioreactor technology (Paek et al., 2005). Since 2002, a biotechnology company in South Korea has been manufacturing ginseng adventitious roots using 10,000 L bioreactors with an average production of 45 tons (FW) per year (Baque et al., 2012b).

Although undifferentiated cell culture has been the focus for bioreactor cultivation studies, a great interest has been shown in organ cultures particularly hairy roots and adventitious roots culture. In some plant species, hairy root cultures are often considered as an efficient alternative method, but it usually produce opine like-substrates that are lethal to mammalian cells (Choi et al., 2000b). Furthermore, the complex structure of hairy root culture makes it difficult to be used as crude extracts (Zhang et al., 2013). This is due to high purification cost of the opine-like substrates compared to field-grown plants. In addition, the major problem associated with hairy root cultures in bioreactors is the restriction of gaseous oxygen delivery to the central mass of tissue, which may results in pocket of senescencing tissue and due to branching; the roots form an interlocked matrix that resists fluid flow. As a result, the bioreactor designs for hairy root cultures need to take into consideration their complicated morphology and shear sensitivity (Giri & Narasu, 2000). Therefore, the ability to exploit hairy root culture as a source of bioactive compounds depends on development of a novel bioreactor system where several physical and chemical constraints associated with these special cultures must be taken into consideration.

Compared to hairy root, opine-like toxic compound does not exist in adventitious root culture (Bhatia & Bera, 2015). Furthermore, the changes of carbon assimilation mode in adventitious root culture have remarkable effects on the patterns of bioactive molecules produced. Therefore, adventitious root culture provides a better system to study the coordination between primary and secondary metabolism (Baque et al., 2010a). Cultivation of adventitious root in large-scale bioreactor is considered as a promising approach for secondary metabolites production of pharmaceutical and nutraceutical interest due to high growth rate and active secondary metabolism (Hahn et al., 2003). Besides, the absence of foreign gene makes adventitious roots as the potential biological material for the production of stable commercially valuable secondary metabolites under *in vitro* conditions (Baque et al., 2010a). In comparison to cell cultures, adventitious roots showed higher stability in their cultivating environment and synthesize enormous amounts of secondary metabolites into their intercellular spaces. Moreover, adventitious root culture can be more easily extracted and grown in a phytohormone amended medium with low inoculum yet producing high growth rate (Sivakumar, 2006). A list of pharmaceutically important medicinal plants studied for biomass or over production of secondary metabolites in a number of bioreactor configurations are depicted in Table 2.80.

Table 2.80: Large scale production of biomass and secondary compound of medicinal plants in different bioreactor configuration and volume.

Plant species	Compound	Bioreactor	Reference
Astragalus membranaceus	Saponin, flavonoid	Balloon type bubble : 5 L	Wu et al., 2011
Azadirachta indica	Azadirachtin	Stirred tank : 3 L	Prakash & Srivastava, 2008
Beta vulgaris	Betalain	Bubble column : 3 L	Savitha et al., 2006
Catharanthus roseus	Ajmalicine, catharanthine	Airlift : 20 L	Zhao et al., 2001
Curcuma zedoaria	Sesquiterpenes	Stirred tank : 10 L	Loc et al., 2009
Echinacea purpurea	Chichoric acid chlorogenic acid, caftaric acid	Airlift : 20 L Balloon type bubble : 500 L; Drum type bubble : 1000 L	Wu et al., 2007
Echinacea purpurea	Caftaric acid, chlorogenic acid, cichoric acid	Ballon type bubble : 5 L	Jeong et al., 2009
Eleutherococcus sessiliflorus	Eleutherosides	Balloon type bubble : 3 L	Shohael et al., 2005
Eleutherococcus koreanum	Eleuthrosides B & E, chlorogenic acid phenolic, flavonoid	Airlift : 3 L	Lee et al., 2015
Eurycoma longifolia	Phenolic, flavanoid	Balloon type bubble : 3 L	Lulu et al., 2015
Hypericum perforatum	Hyperecin	Balloon type bubble : 3 L	Cui et al., 2010a
Panax ginseng	Saponin, ginsenoside	Balloon type bubble : 5 L, 20 L, 500 L	Choi et al., 2000b
Panax ginseng	Ginsenoside	Balloon type bubble : 5L Balloon type : 10 000 L, 20 000 L	Jeong et al., 2006
Panax ginseng	Ginsenoside	Airlift : 5 L and 20 L	Paek et al., 2009
Podophyllum hexandrum	Podophyllotoxin	Stirred tank : 3 L	Chattopadhyay et al. 2002
Scopolia parviflora	Alkaloid	Column : 1L	Min et al., 2007
Vitis vinifera	Stilbenes, catechins	Stirred tank : 1 L	Ferri et al., 2011

CHAPTER 3: ESTABLISHMENT OF CELL SUSPENSION CULTURES IN SHAKE FLASK SYSTEM AS A SOURCE OF BIOACTIVE COMPOUND

3.1 INTRODUCTION

3.1.1 Plant secondary metabolites

Plant secondary metabolites are extremely diverse with thousands identified in several major classes, for human use as medicines, flavorings, or recreational drugs. Plant secondary metabolites are generally classified according to their biosynthetic pathways. There are three large molecule families namely phenolics, terpenes & steroids, and alkaloids (Bourgaud et al., 2001). Secondary metabolites could also be classified based on the presence of nitrogen. The nitrogen containing secondary metabolites which includes, alkaloids, non-protein amino acids, amines, cyanogenic glycosides and glucosinolates. Whereas the one without nitrogen; are terpenoids (mono, sesquiterpene, diterpenes, triterpenes, steroids and saponines) and phenolics (flavonoids, polyacetylens, polyketides and phenylpropanoids).

3.1.2 Sesquiterpenoids

Sesquiterpenoid or sesquiterpene is a class of terpenes that consist of three isoprene units and a 15-carbon backbone with a molecular formula $C_{15}H_{24}$. Sesquiterpenoids are found abundantly in plants from the family Asteraceae, which have a highly significant role in human health. Their functions include both as part of a balanced diet and as pharmaceutical agents, attributable to their major potential of cardiovascular disease and cancer treatment (Chadwick et al., 2013). Sesquiterpenoids are typically located in laticifers, a specialized secretory cell in most of the Asteraceae. Nevertheless, it can also be found within the vacuoles of other cell types, specifically when produced in response to biotic stresses. Sesquiterpenoids are one of the main constituents of latex in latex

producing plants, and they act as powerful antimicrobial agents as well as antifeedants to chewing insects and birds (Chadwick et al., 2013). In addition, they also have other effects such as allelopathy, stimulation of germination in certain parasitic herbaceous plant (De Luque et al., 2000), and showing species and compound dependent modification of root growth in *Lactuca sativa* L., *Lepidium sativum* L., *Solanum lycopersicum* L., *Hordeum vulgare* L., and *Triticum aestivum* L. (Macías et al., 1996).

Sesquiterpenes are colorless lipophilic compounds and the biosynthesis in plants is from three isoprene units, which occurs via farnesyl pyrophosphate (FPP), in the endoplasmic reticulum (Yu & Utsumi, 2009). The large number of sesquiterpene synthases (Bennett et al., 2002; Cheng et al., 2007) in addition to the fact that a single synthase may produce several products and further modifications after sesquiterpene synthesis, such as oxidation and glycosylation (Little & Croteau, 2002; Schnee et al., 2002) resulted in a vast number of varied structures. Several similar syntheses may produce identical products, in different ratios which may affect the metabolite profile of a plant and can be utilized to classify closely related species or subspecies (Chadwick et al., 2013).

3.1.2.1 Zerumbone

Zerumbone (ZER) is a sesquiterpene with monocyclic or one ring with fifteen carbons and 220 hydrogen ($C_{15}H_{220}$). The molecular weight of zerumbone is 218.340 unit and is a construct of fifteen carbons with one double bond oxygen at r-chain (Figure 3.1). There are several IUPAC name for ZER such as (E,E,E)-2,6,9,9-Tetramethyl-2,6,10-cycloundecatrein-1-one, 2,6,10-Cycloundecatrein-1-one, 2,6,9,9-tetramethyl-, and (E,E,E)-471-05-6. ZER can be classified as an intermediate between polar and non-polar compound.

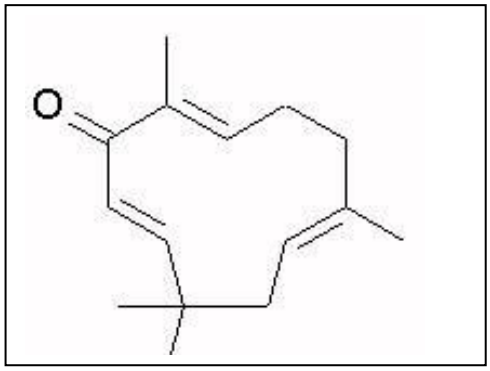

Figure 3.1: Chemical structure of zerumbone.

3.1.2.2 Biosynthetic pathway of zerumbone

The first committed step to formation of ZER is the cyclization of (2E,6E)-farnesyl diphosphate through terpene synthase forming (E)-ß-cryophyllene and α-humulene. Yu et al. (2011) proposed the ZER biosynthesis was based on the presence of α-humulene and a trace amount of 8-hydroxy-α-humulene in rhizome oil of *Z. zerumbet*.

The initial step in ZER biosynthesis is catalyzed by α-humulene synthase (ZSS1), which cyclizes the universal sesquiterpene precursor farnesyl diphosphate (FPP) to α-humulene to establish the basic skeleton. This parent olefin is thought to being hydroxylated at the C8 position by a region specific cytochrome P450 enzyme and then oxidized by an alcohol dehydrogenase to generate ZER. The difference between α-humulene and ZER is the presence of the carbonyl moiety in ZER (Yu et al., 2008). The biosynthesis pathway of ZER is shown in Figure 3.2.

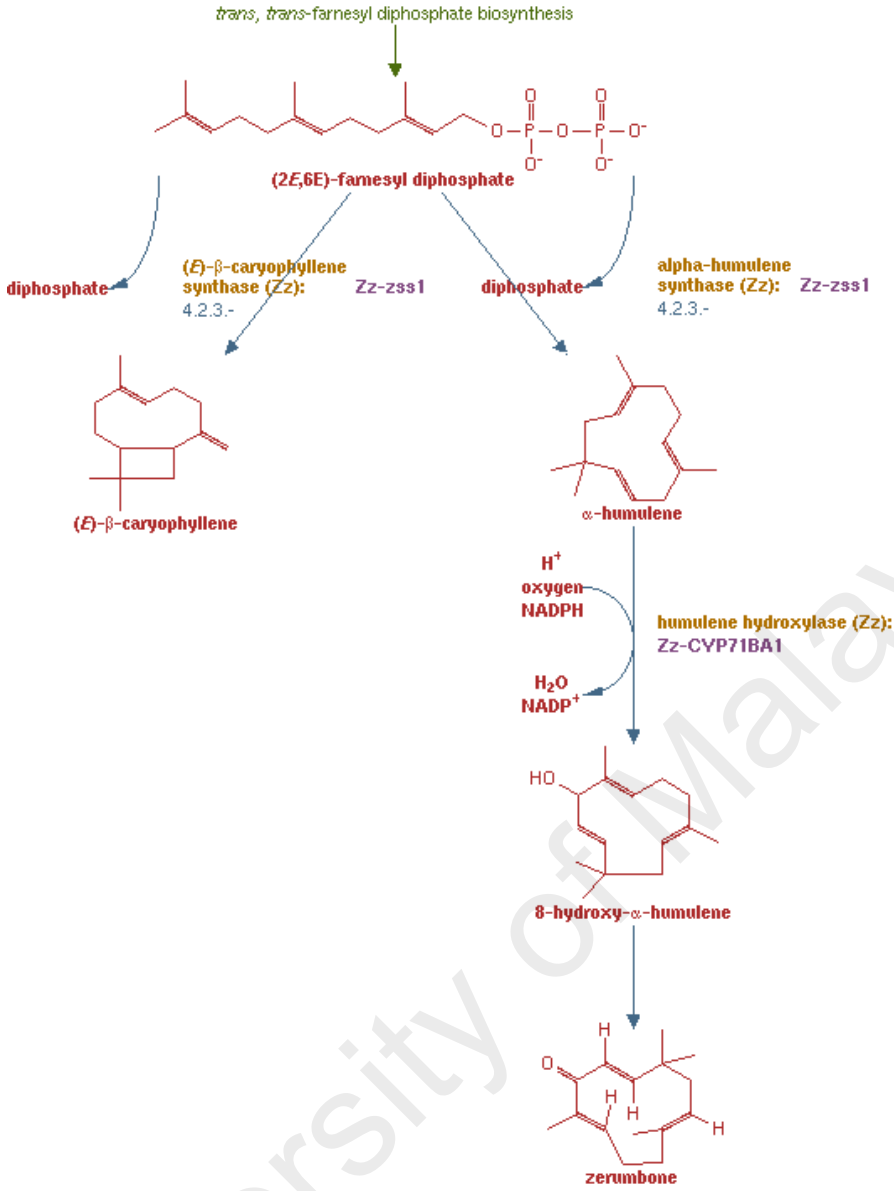

Figure 3.2: Biosynthetic pathway of zerumbone (Yu *et al*., 2011).

3.1.3 Bioactive compounds in *Zingiber zerumbet* Smith.

Zingiber zerumbet Smith is a perennial edible ginger with high phytomedical properties, commonly being used as herbal medicine in Asian, Indian, Chinese and Arabian communities ever since primeval time (Yob et al., 2011). The bioactive compounds in *Z. zerumbet* are normally found in the essential oil and it can be extracted from all parts of the plant particularly the rhizomes, leaves as well as flowers (Chane-Ming et al., 2003). The major compounds which can be found in the rhizomes are zerumbone (ZER) (37%) followed by alpha-humulene (14%) and camphene (13.8%)

(Sulaiman et al., 2010). Leaves and flowers produce different types of compounds from rhizomes with (E)-nerolidol as the major compound and also beta-cryophyllene and linalool (Bhuiyan et al., 2009).

Studies by Jang et al. (2004) reported on the isolation of aromatic compounds and kaempferol derivatives from the $CHCl_3$-soluble fraction of the MeOH extract of the rhizome of *Z. zerumbet* while recently they discovered potentially new bioactive sesquiterpenoids namely 6-methoxy-2E,9E-humuladien-8-one (1 & 2) and stigmast-4-en-3-one (Jang & Seo, 2005). Other studies by Yu et al. (2008) stated that the combination of GC and GC/MS analysis on rhizome oil disclosed the occurrence of over 16 sesquiterpeniods consisting of high percentage of oil (85.81%), with the remaining 24.19% of monoterpenoids (Koga et al., 2016). The oil was characterized by the presence of zerumbone (48.13%), a-humulene (17.23%), humulene epoxide I (7.88%) and humulene epoxide II (5.74%), accounting for 92% of the total sesquiterpenoid fraction while b-caryophyllene (1.15%), caryophyllene oxide (3.18%), b-eudesmol (0.21%) and other minor sesquiterpenoid components were also identified (Koga et al., 2016).

ZER has been intensively studied for its potential use as anti-inflammatory, antifungal, chemopreventive and chemotherapeutic compound (Murakami et al., 2002; Jantan et al., 2003). ZER has been found to suppress tumor promoter cells (Murakami et al., 2002; Koga et al., 2016) and also could inhibit the growth of human leukemia cell line, HL-60 cell (Huang et al., 2005). A study conducted by Chang et al. (2012) and Chia et al. (2014) discovered that the ethanol extract of *Z. zerumbet* is not mutagenic and safe with respect to genotoxicity and general toxicity if individuals are provided with a proper dose. It was also figured out that ZER could be used as chemopreventive

agent in hepatocarcinogenesis (Taha et al., 2010) while Zhang et al. (2012) proved that ZER induced apoptosis in pancreatic carcinoma cells.

3.1.4 High performance liquid chromatography (HPLC)

HPLC is a chromatographic technique used in compounds mixture separation in analytical chemistry and biochemistry with the purpose of identifying, quantifying and purifying the individual components of the mixture. HPLC has various applications such as chemical separations, purification, identification and quantification. Preparative HPLC refers to the process of isolation and purification. Analytical HPLC focuses on obtaining information on identification, quantification and resolution of the samples.

3.2 MATERIALS AND METHODS

3.2.1 Plant materials

The rhizomes of *Z. zerumbet* or Lempoyang (herbarium number 041148) were obtained from a nursery at Pusat Asasi Sains, University of Malaya. The rhizomes were then introduced to a 'forced growth' in the lab by regular sprinkling of water to produce shoot buds. The sprinkling of water was done sparingly to induce shoots and at the same time avoiding the rhizome from being too wet causing fungal contamination. After 2-3 weeks of forced growth, small buds sprouted and buds at 1.5-2.0 cm in length were harvested to be used as explant.

3.2.2 Sterilization procedure

3.2.2.1 Explant sterilization

Surface sterilization was done on the shoot buds obtained from the 'forced growth'. The shoot buds were excised from the rhizome and washed with tap water to remove dirt. After cleaning the shoot buds, excessive roots and leaves were trimmed. Then, the

shoot buds were soaked in distilled water added with two to three drops of Tween 20 for about 30 minutes. The purpose of adding Tween 20 is to reduce the surface tension so that the solution for sterilization can reach the surface of the explants. Shoot buds were then rinsed under running tap water and subsequently submerged into 70% (v/v) Clorox (5.25% of sodium hypochlorite) solution with a few drops of Tween 20. After thirty minutes, the shoot buds were removed and placed aseptically in a solution of 20 % (v/v) Clorox and another few drops of Tween 20. Subsequently, after 10 minutes the shoot buds were rinsed three times with sterile distilled water and air-dried on sterilized filter paper in the laminar air flow chamber. The sterilized shoot buds were then excised to 1.0 cm in length and ready for culture.

3.2.2.2 Sterilization of media and apparatus

All the culture media and apparatus were sterilized in autoclave at 121°C and pressure of 15 psi. The time required for autoclaving the culture media, distilled water and other apparatus such as forceps, scalpels, cutting plates and glassware were 20 minutes. The indicator tape was used to assist in determining the completion of the sterilization procedure.

3.2.3 Establishment of cell suspension cultures and growth conditions

3.2.3.1 Cell suspension culture initiation and culture condition

The callus of *Z. zerumbet* was initiated by culturing slices of shoot buds on Murashige and Skoog (MS) basal medium (Appendix A) supplemented with 1.0 mg/L d-biotin, 2.0 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), 1 mg/L indoleacetic acid (IAA), and 1 mg/L 1-naphthaleneacetic acid (NAA) (Idris et al., 2010). Cells suspension cultures were established by culturing friable callus in liquid MS medium containing 1mg/L 2,4-D, 0.1g/L malt extract, 0.1 g/L glutamine, 250 μL/L zeatin, and

20 g/L sucrose. The media were adjusted to 5.7 prior to autoclaving. All cultures were maintained at 25 ± 1°C on orbital shaker at 70 rpm under a photoperiod of 16 h light and 8 h dark cycle with a light intensity of 31.4 μmol m^{-2} s^{-1} provided by fluorescent lamps. The cell suspensions were subculture for every 7 days to multiply the cells. Newly established cell suspension cultures were used as material in optimizing chemical and physical parameters for growth and compound harvesting.

To optimize growth conditions for suspension cultures, carbohydrate substrates (2% sucrose, maltose, fructose, and glucose), and different concentrations of sucrose (1%, 2%, and 3%) (w/v), pH of medium (pH 5.2, 5.7, and 6.2), light regime (16 h light : 8 h dark and dark condition), agitation (40 rpm, 70 rpm, and 100 rpm), and incubation temperature (18, 24, and 30°C) were tested. The cells with initial volume of 0.5 mL settled cell volume (SCV) were inoculated into liquid MS media according to the experimental objectives. All experiments were carried out in triplicate cultures and each treatment was repeated three times.

3.2.3.2 Determination of cell biomass

Determination of cell biomass was done through the measurement of settled cells volume (SCV). The fraction of the cell in liquid media was measured using graduated 15 mL Falcon tubes. The culture was transferred aseptically into Falcon tube and the cells were allowed to settle via gravity for 5-10 min.. The growth of cell suspension cultures was measured by SCV every 7 days for a period of 42 days whereas the specific growth rate (the changes of SCV in natural log) was recorded for 28 d of culture. All treatments are subjected to 2% (w/v) of sucrose except for treatment with carbohydrate substrate, which tested three other carbohydrate types. All media were adjusted to pH 5.7 prior to autoclaving and culture under continuous shaking condition

at 70 rpm. All experiments were carried out in triplicate cultures and each treatment was repeated three times.

3.2.4 HPLC instrument setup and solvent gradient configuration

The Waters HPLC system consisted of a W600E multisolvent delivery system, W2489UV/visible detector, W2707 auto sampler and in-line de-gasser. The system was controlled by Empower 2 software. The guard and reverse (Chromolith RP-18 encapped, 100-4.6 mm) used were from Merck. HPLC grade methanol (Merck Ltd.), HPLC grade acetonitrile (Fisher Ltd.) and HPLC grade phosphoric acid (BDH Ltd.) were used in HPLC analysis. Water used for preparation of the phosphoric acid solution was purified using Millipore filter. The injection volume was 20 µL, and the eluent was observed at wavelength, λ: 254 nm. The solvents used for elution was 0.1% (v/v) phosphoric acid (A) and acetonitrile (B). The guard column and column were flushed with pure HPLC grade acetonitrile before and after usage.

3.2.5 Establishment of standard calibration of compound using HPLC

ZER from Sigma, USA was used as standard. The retention time of standard ZER was obtained from HPLC chromatogram at 10.6 min. The solvent used to prepare all the solutions was acetonitrile (ACN) with ZER concentration 0.04 mg/ml, flow rate of 1 µL/min, running time 10 minutes and temperature 25°C. Standard calibration comprising the ZER was developed at λ = 254 nm. Six levels of injection volumes were chosen that is 1.0, 3.0, 5.0, 7.0, 9.0 µL. Triplicate reading was applied. From the HPLC chromatograms, the peak area of each dilution for each standard compound was determined and a standard calibration was obtained for ZER.

3.2.6 Preparation of extract for analysis of zerumbone

3.2.6.1 Rhizome

The rhizomes of *Z. zerumbet* were cleaned, sliced and oven dried at 38°C for 48 h. The oven-dried samples were pulverized to powder then sieved by using a metal sieve. The finely ground samples were weighted (0.1 g) and extracted by using Soxhlet method (3.2.7.2). The crude extract was later evaporated by using rotary evaporator (BÜCHI Rotavapor R-114). The extract was dissolved in 10mL dichloromethane (DCM, Merck, USA) and kept at 4°C in the chiller until use. Each analysis was repeated three times.

3.2.6.2 Cell suspension cultures

The cell was collected after 15 days of initiation and oven-dried for 48 h at 38°C. The finely ground powder (0.1 g) was extracted using Soxhlet method for cells masses and 30 mL of filtrate was subjected to partitioning. The crude extract was later evaporated by using rotary evaporator (BÜCHI Rotavapor R-114). The extract was dissolved in 10 mL dichloromethane (DCM, Merck, USA) and kept at 4°C in the chiller until use. Each analysis was repeated three times. The mixture containing compound of interest was filtered through 0.45μm PTFE filter (Sartorius 13 CR) for HPLC analysis.

3.2.7 Methods for compound extraction

3.2.7.1 Partitioning for liquid extraction

The cells suspension was filtered by using filter paper (Whatman, 9 cm) and the filtrate was collected. Partitioning or liquid-liquid extraction was used to obtain the crude extract from spent liquid medium of cells suspension culture. The solvent used for partitioning of *Z. zerumbet* suspension cultures was DCM AR grade. Spent medium of

cell suspension were measured at 30ml and poured into 500 mL separating funnel. An equal volume (150 ml) of distilled water added with sodium chloride (NaCl) and DCM AR grade were then poured into the same funnel. The sodium chloride was used to polarize the distilled water.

The mixture of the spent liquid medium, distilled water and DCM were then shaken vigorously to make sure the polar and non-polar compound were well-separated. Shaking caused pressure inside the separating funnel to be increased. To release the pressure, the funnel was placed in an inverted position and the stop cock was opened to release the pressure. The process of shaking was repeated several times then it was left to separate into two layers. The lower part consisted of DCM with non-polar compounds while the upper part contained distilled water and the polar compound. The lower layer was collected whereas the upper aqueous layer was discarded. Sodium nitrate anhydrous was added to the collected DCM dissolved extract to absorb residual water in the extract and later kept overnight. After an overnight incubation, the extract was then poured into a round flask and evaporated using rotary evaporator. The dried extract was then kept in 10 ml DCM stock. Stock was ready for further analyses using HPLC.

3.2.7.2 Soxhlet for cell biomass extraction

The Soxhlet extractor is a laboratory apparatus originally designed for extraction of lipid from solid material invented by Franz von Soxhlet in 1879. However, the usage of the Soxhlet extractor was not limited to the extraction of lipid. Other compounds which remain stable during temperature changes can also be extracted using the Soxhlet extractor. Soxhlet extractor includes the condenser, thimble with thick filter paper, heating instrument and round flask where the extract was collected. The dried sample

about 0.1 g was put in the thick filter paper inside the thimble. The boiling point of DCM is low which is at 39.8°C so the soxhlet extractor was set to a low temperature at 40-50°C for a period of six hours. Heat promoted the higher solubility and along with a relatively low boiling point, this technique is suitable for the extraction of ZER.

3.2.8 Solubility of standard zerumbone (Sigma) in different solvents

Compound extraction was done according to procedure in section 3.2.71. The dried extract was kept in 10 ml DCM stock. The stock was dissolved in three different solvent for solubility test. The solubility of ZER has been tested in three different solvents namely water, methanol and acetonitrile. The samples were prepared in triplicates and 6 µl samples were loaded in HPLC for analysis.

3.2.9 Identification of zerumbone compound

The ZER compound was identified by matching its retention times (10.6–10.8 minutes) and spectral characteristics to a commercially available standard ZER (Sigma, USA). When there was uncertainty, the extract was spiked with a mixture of standards. The retention times, spectral characteristics and intensities of the peaks of the resulting chromatograms with and without spiking were compared, and the compound was identified. The concentrations of compounds were calculated as follows:

For cells suspension/root/rhizome:

$$\text{Zerumbone } (\mu g/g) = \frac{\text{Peak area of samples}}{\text{Peak area of Standard area}} \times \text{Standard concentration } (\mu g/ml) \times \frac{\text{Extract volume (ml)}}{\text{Dry weight (g)}}$$

3.2.10 Statistical analysis

All data collected were analyzed by one-way ANOVA followed by Tukey's HSD test at a significance level of $p < 0.01$.

3.3 RESULTS AND DISCUSSION

3.3.1 Establishment of cell suspension cultures

After 6 weeks of culture, friable callus (Figure 3.3A) obtained from the initial culture of shoot buds slices was transferred into liquid medium for initiation of cell suspension culture. Heterogeneous cells containing dense cytoplasm were observed within one month of culture. The cells were filtered and continuously sub-cultured. Homogeneous cell suspension cultures with dense yellowish cytoplasm were obtained after 2-3 months of culture (Figure 3.3B). The morphology of cells suspension of *Z. zerumbet* was similar to those of viable cells reported in banana cell suspension culture (Jalil et al., 2008).

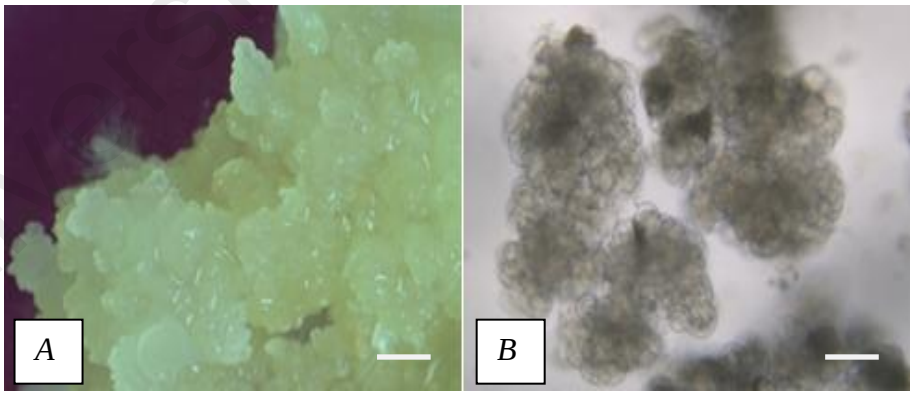

Figure 3.3: Initiation of cell suspension culture of *Z. zerumbet*; *A* : friable callus, Bar : 1mm; *B* : cell suspension culture, *Bar : 100µm*.

3.3.2 Solubility of zerumbone (Sigma) in different solvents

Preliminary work to determine the solvent system for ZER was carried out using commercial standard. The solubility test on ZER showed that the acetonitrile presented the highest solubility of 58.35 ± 2.48 mg/L comparatively. ZER was less soluble in water with 10 fold lower than acetonitrile (Figure 3.4). As a result, acetonitrile was chosen as the solvent in the subsequent HPLC analysis for compound extraction from rhizome and cells suspension cultures due to cost and quality of the compound.

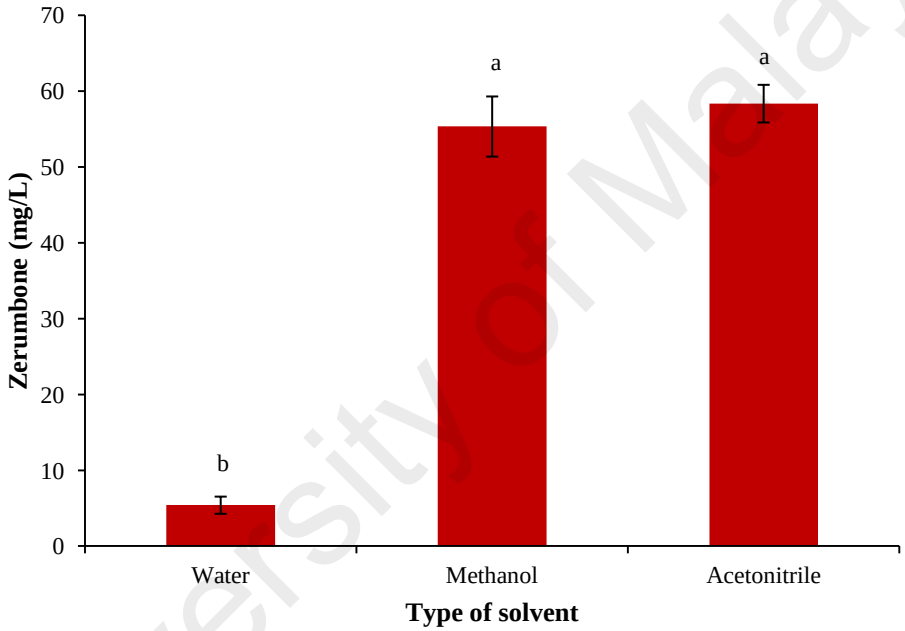

Figure 3.4: Solubility test of zerumbone for HPLC analysis.

3.3.3 Establishment of standard calibration curve

The standard curve of ZER was calibrated at λ = 254 nm for determination of ZER concentration in the subsequent experiments (Figure 3.5). HPLC analysis showed that the retention time for ZER was at 10.6 minutes (Figure 3.6). This retention time will be used to determine the ZER peak in the HPLC chromatogram obtained.

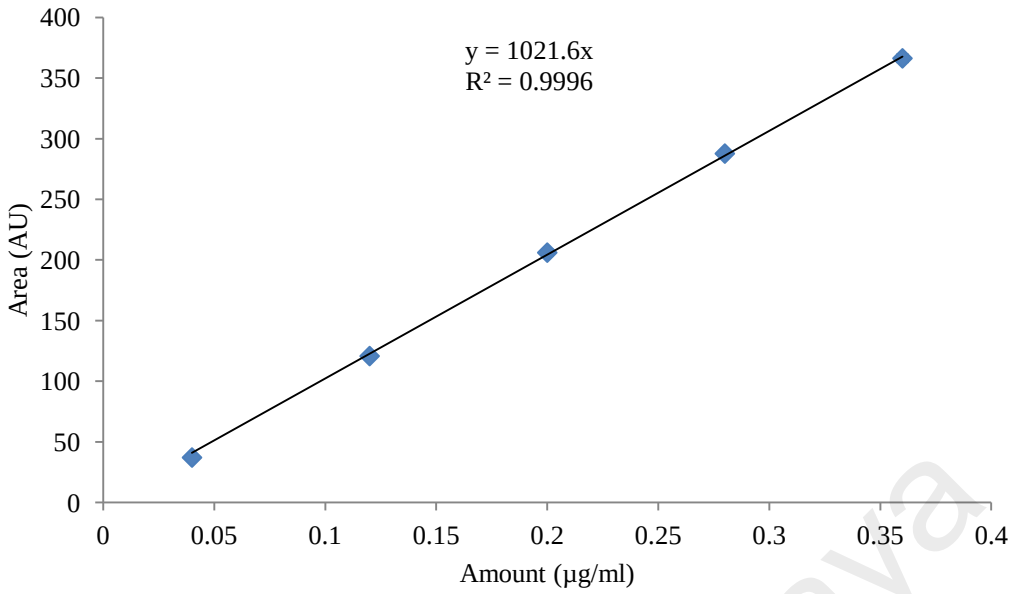

Figure 3.5: Standard calibration curve of zerumbone (Sigma, USA)

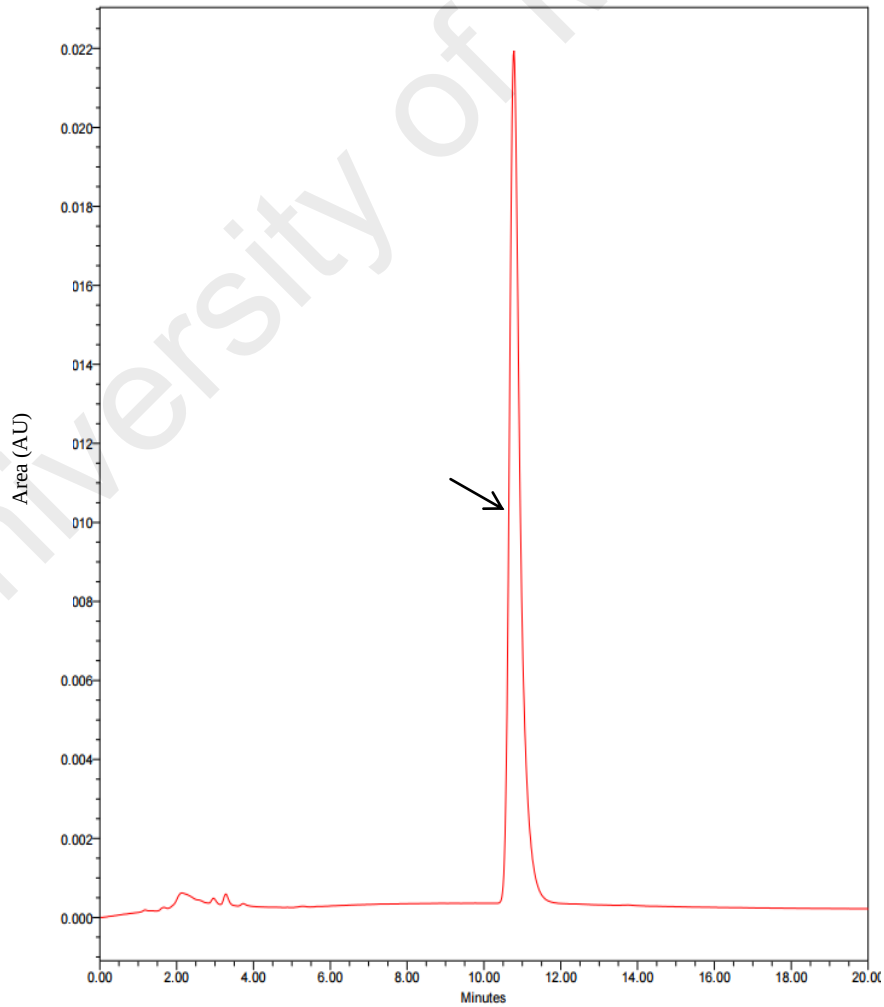

Figure 3.6: Chromatogram of standard zerumbone (Sigma).

3.3.4 Zerumbone compound accumulation and quantification in rhizome of *Z. zerumbet*

The HPLC analysis of rhizome extract of *Z. zerumbet* showed the occurrence of ZER compound. The retention time was at 10.6 minutes (Figure 3.7).The concentration of ZER is 29,900 ± 1700 µg/g DW.

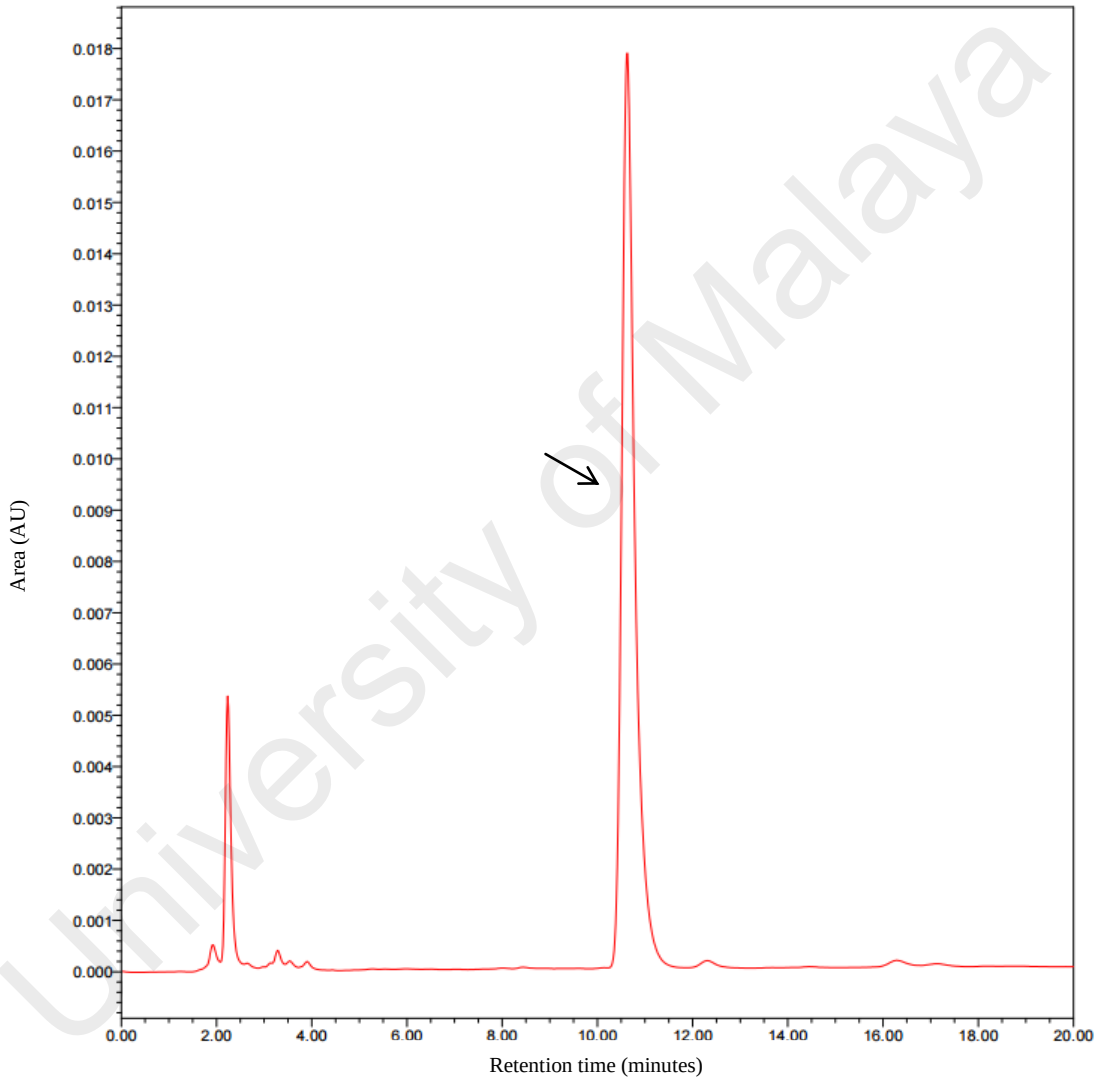

Figure 3.7: Chromatogram showing detection of ZER compound in the rhizome of *Z. zerumbet.*

3.3.5 Zerumbone compound accumulation and quantification in cell suspension cultures of *Z. zerumbet*

One of the major uses of plant cell cultures is for the production of valuable bioactive products for pharmaceutical, flavours, fragrance and fine chemicals. One of the advantages of using cell culture in producing secondary metabolites is accumulation of product yield higher than the parent plants (Hippolyte et al., 1992; Estrada- Zúñiga et al., 2009; Idris et al., 2009). Nonetheless, in dealing with cell cultures, condition needs to be optimized in order to get maximum yield. The growth and capability of producing bioactive compound in cell cultures was believed to be related with chemical and physical parameters. The specific growth rate of all the parameters tested is shown in Table 3.1.

In this study, although growth conditions of cell suspension have been optimised, the cell biomass showed no ZER accumulation. However, ZER was found in the spent liquid medium. The HPLC profile was shown in Appendix B and C respectively. This might be due to secretion of ZER compound into the surrounding medium or cell lysis. This finding was in contrast with *Boesenbergia rotunda* cell suspension culture in which the bioactive compounds were accumulated in cell biomass (Yusof et al., 2013). Zhang et al. (2002) stated that in plant cell cultures, the secondary metabolites are synthesized either by secretion into the surrounding medium or stored intracellularly.

Table 3.1: Specific growth rate of *Z. zerumbet* cell suspension cultures.

Parameters		Specific growth rate ($x\ 10^{-2}\mu \pm SE$)
Carbohydrate substrates	Sucrose	7.14 ± 0.12^{a}
	Glucose	4.81 ± 0.11^{d}
	Fructose	2.44 ± 0.12^{g}
	Maltose	$5.93 \pm 0.12^{b,c}$
Sucrose concentrations	10 g/L	3.85 ± 0.10^{e}
	20 g/L	7.15 ± 0.09^{a}
	30 g/L	7.45 ± 0.12^{a}
pH	5.2	6.09 ± 0.09^{b}
	5.7	7.07 ± 0.01^{a}
	6.2	$5.29 \pm 0.24^{c,d}$
Light regime	16 light : 8 dark	7.16 ± 0.10^{a}
	dark	2.38 ± 0.17^{g}
Agitation	40 rpm	0.91 ± 0.77^{h}
	70 rpm	7.05 ± 0.27^{a}
	100 rpm	$5.76 \pm 0.13^{b,c}$
Temperature	18°C	1.66 ± 0.23^{h}
	24°C	6.31 ± 0.21^{b}
	30°C	$3.31 \pm 0.06^{f,e}$

The data collected were analyzed by ANOVA and the mean values were tested to evaluate the significance at the 1% confidence level using Tukey's HSD test.

3.3.5.1 Effect of carbohydrate on biomass growth and zerumbone production

Carbohydrate is one of the most important elements supplied into the media for the *in vitro* plant cells growth. In this study, we observed that the growth of cell suspension cultures was influenced by the carbohydrate substrate (Figure 3.8a). Of these, cells cultured in sucrose-based medium recorded the highest specific growth rate ($7.14 \pm 0.12^{a} \times 10^{-2}\mu$) and ZER content ($3.73 \pm 0.61$ mg/L), followed by maltose and glucose (Figure 3.8b). Low specific growth rate and ZER content were observed when cells were cultured in medium supplemented with fructose, indicating that fructose was less preferred for cell growth.

Different carbon sources have been reported to influence the callogenesis and organogenesis (Vespasiano & Otoni, 2003). Petersen et al. (1999) stated that sucrose is the main carbon supply among the available carbon sources. However, it may cause hypoxia and ethanol accumulation in the cells as a result of its quick metabolization (Scott et al., 1995; Ramarosandratana et al., 2001). Therefore, in some cases to promote cell growth, sucrose was wholly or partially replaced by other carbon sources (Rout et al., 2000). Kim et al. (1995) established optimum production of paclitaxel from *Taxus brevifolia* cell suspension cultures in medium containing fructose as the carbon source and similarly, the production of catharantine in *Catharanthus roseus* cell cultures was doubled when fructose was used as a carbon source (Kim, 2001)

In our study, the production of ZER was also significantly affected by different carbohydrate substrate. This was in accordance with findings by Do and Cormier (1991) where carbon source has affected the accumulation of anthocyanins in *Vitis vinifera* cell suspensions. Similar observation was made on *Lithospermum erythrorhizon* cell cultures for the production of shikonin (Srinivasan & Ryu, 1993). This proved that the

carbohydrate substrate is one of the parameter need to be consider for secondary metabolite harvesting from cell culture.

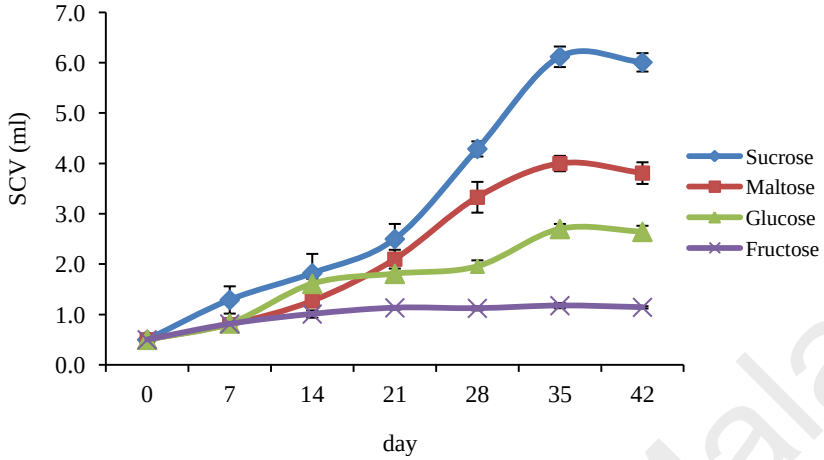

Figure 3.8a: Cell growth in different carbohydrate substrate.

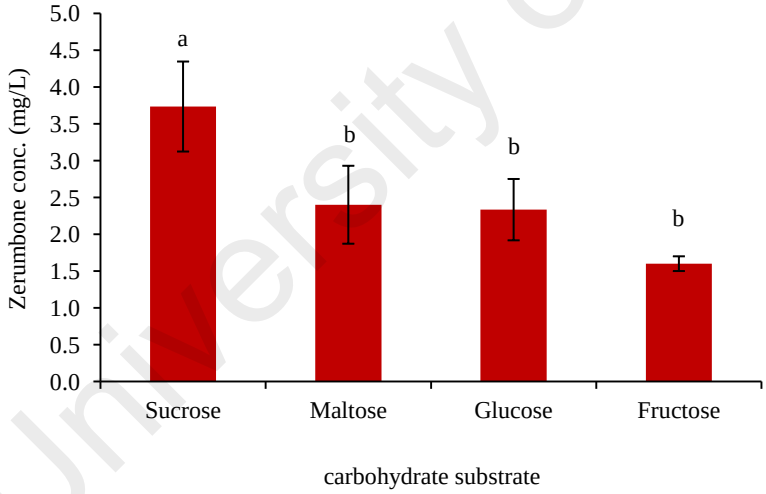

Figure 3.8b: Zerumbone production in different carbohydrate substrate.

3.3.5.2 Effect of sucrose concentration on biomass growth and zerumbone production

In the subsequent experiment, we tested MS medium with different concentrations of sucrose. Cells cultured in medium containing 2 and 3% sucrose showed almost similar specific growth rates (7.15 ± 0.09 and 7.45 ± 0.12 x 10^{-2}μ, respectively) with doubling time at about 5-6 days of culture whereas medium with 1% sucrose showed the lowest growth rate with doubling SCV at 15-20 days of culture (Figure 3.9a). The 2 and 3 % sucrose concentration showed no significant differences in terms of cell growth but 1% sucrose significantly affect the biomass accumulation. Sucrose served as a carbon source and regulator in cells osmosis (George, 1993). Most *in vitro* cultures are autotrophic-incompetent and are not able to proliferate properly without exogenous supply of carbohydrates (Cournac et al., 1991). For the regeneration of *Digitalis lanata*, the optimum concentration for all the carbohydrates tested (sucrose, maltose, fructose, and glucose) was 3.0% (Fatima et al., 2009). However, too high sucrose levels might cause cells dehydration and therefore reduced cells proliferation. This fact was supported by findings that stated high sucrose concentration, would reduce the shoot length in *Curcuma xanthorriza* and *Zingiber aromaticum* regeneration (Kusumastuti et al., 2014).

The highest amount of ZER (3.70 ± 0.05 mg/L) was detected on medium containing 3% sucrose (Figure 3.9b). For the production of ZER, it was not significantly affected by the difference in sucrose concentrations. This might be due to the range of sucrose studied is still within the acceptable concentration and did not result in osmotic stress to the cells. This was evidenced in *Hypericum perforatum* root culture, where too high sucrose concentration (5, 7 and 9%) inhibited the biomass accumulation of root culture but enhanced the amount of total phenols, flavonoids, chlorogenic acids and total hypericin content (Cui et al., 2010b). In *Rubia tinctorum* cultures, 12% sucrose

produced maximum anthraquinone production (Sato et al., 1991). So the concentration of carbon source needs to be optimized in order to produce high bioactive compound.

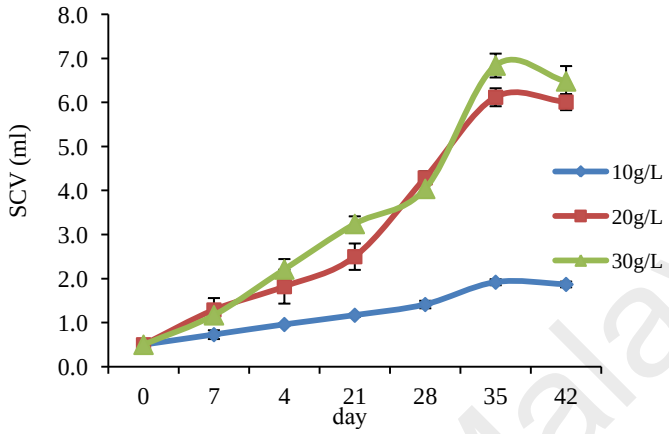

Figure 3.9a: Cell growth in different sucrose concentrations.

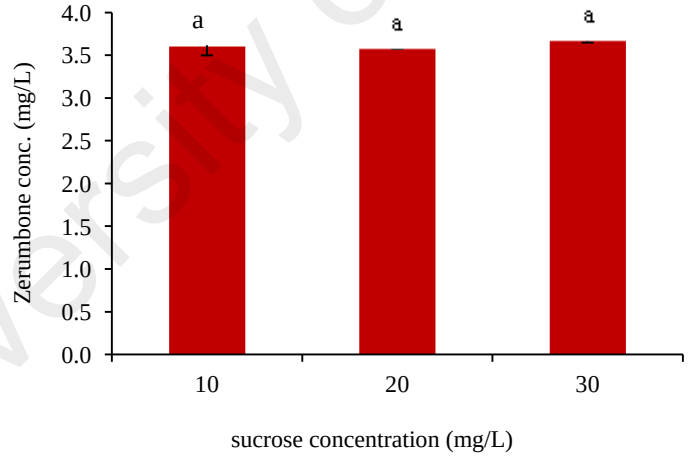

Figure 3.9b: Zerumbone production in different sucrose concentrations.

3.3.5.3 Effect of light regime on biomass growth and zerumbone production

Light regime is another factor affecting the cell growth and ZER production. Cells incubated under 16 : 8 (light : dark) condition double the initial SCV on day 5 and produced the highest amount of ZER (3.42 ± 0.54 mg/L), whereas, under dark condition, SCV were only doubled on day 28 and produced 2.53 ± 0.46 mg/L ZER (Figures 3.10a and 3.10b). In this study, both biomass and bioactive compound accumulation in *Z. zerumbet* cell culture was significantly affected by the light regime. It was proven that irradiation had a remarkable effect on the growth of *Oldenlandia affinis* callus (Dörnenburg & Seydel, 2008) while other report showed that the light quality played an important role in the growth somatic embryo in *Carica papaya* L. (Ascencio-Cabral et al., 2008). For the induction of rooting in *Eucalyptus saligna* Smith and *Eucalyptus globulus* Labill, the dark condition was required in the initial phase to increase the efficiency of the process (Corrêa et al., 2005). In contrast, *in vitro* rooting of most conifers prefers a 16 h photoperiod (Parasharami et al., 2008).

As for compound production, Baque et al. (2010b) reported in *Morinda citrifolia* adventitious root culture, light quality significantly affected the biomass and the total contents of anthraquinone, phenolic, and flavonoid. While in other species the effect of light on the accumulation of biomass and metabolites production was contradict. In *Hyoscyamus muticus* cell suspension culture, light condition even though promotes better growth; the alkaloid content in contrast was suppressed (Aly et al., 2010).

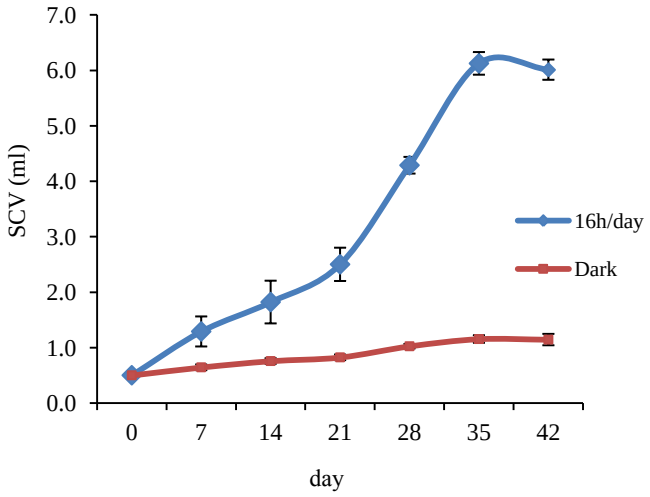

Figure 3.10a: Cell growth in different light regime.

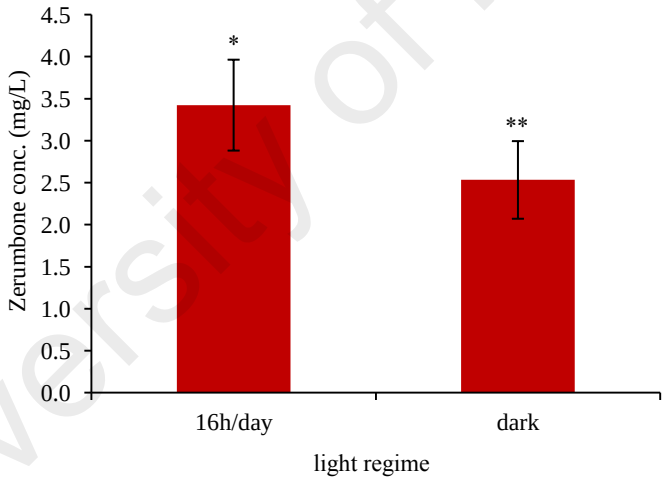

Figure 3.10b: Zerumbone production in different light regime.

3.3.5.4 Effect of temperature on biomass growth and zerumbone production

Temperature ranging from 17 to 25°C is normally applied for callus induction and growth of plant cell cultures. In this study, we also investigated the effect of temperature on cell growth and ZER production.

Our results revealed that low temperature (18°C) negatively affected the cell growth and ZER production (Figures 3.11a and 3.11b). Only 1.87 mg/L ZER was detected in cells incubated at 18°C. At 24°C, cell growth was drastically increased after 21 days of culture with production of 3.90 mg/L ZER. An increased in temperature (30°C) significantly elevate ZER accumulation (4.03 ± 0.35mg/L). This result was in accordance with study by Choi et al. (2000) that even though temperature of 24°C promoted the cell growth but for optimum paclitaxel production a higher temperature at 29°C is needed. However, contrasting finding was obtained by Toivonen et al. (1992) which stated that decreasing of cultivation temperature elevated the total fatty acid content per cell (DW) in *Catharanthus roseus* cells suspension culture. Cell suspension cultures of strawberry also displayed the highest anthocyanin content when cultivated at 15°C in comparison to the one cultured at 35°C (Zhang et al., 1997). This proved that temperature played an important role in biomass and compound extraction from cell culture.

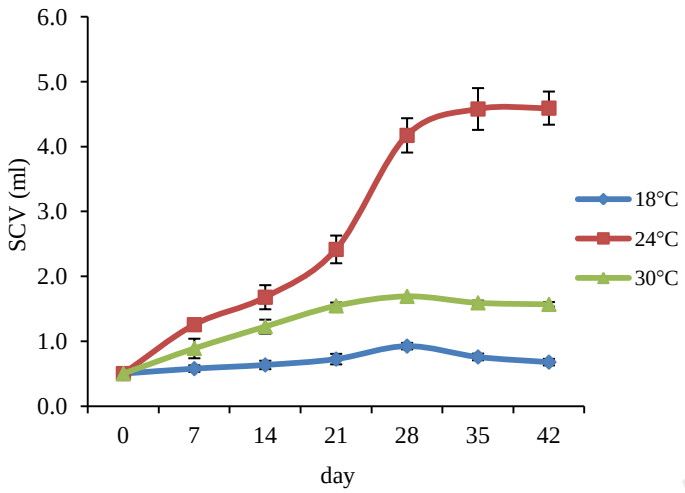

Figure 3.11a: Cell growth in different agitation in different incubation temperatures.

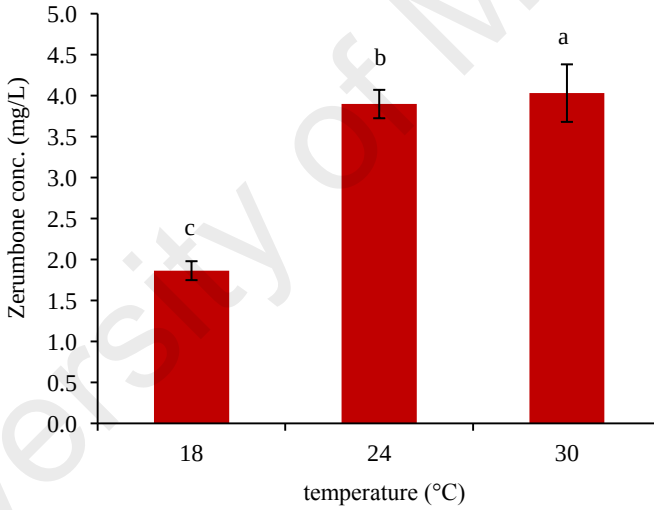

Figure 3.11b: Zerumbone production in different incubation temperatures.

3.3.5.5 Effect of agitation on biomass growth and zerumbone production

In cell culture systems, shear forces produced by agitation can cause several physiological and morphological variations including cell size, shape, aggregation, cell wall composition, integrity, viability, and might ultimately affect biomass accumulation and secondary metabolism (Zhao & Verpoorte, 2007). Agitation of cell cultures mixes cells with the liquid medium and subsequently facilitates nutrient uptake. Therefore, optimal agitation speed plays an essential role in producing excellent biomass growth and secondary metabolite production.

In this study, agitation at 70 rpm and 100 rpm was found to support the growth of *Z. zerumbet* cell suspension cultures. Minimum cell growth was observed when agitated at 40 rpm compared to others (Figure 3.12a). In contrast, for *Boesenbergia rotunda* cell suspension cultures, the lowest yield of final dry weight was from cultures agitated at 120 rpm which displayed vacuolated cells and light brown in color an indicator of low robustness (Yusof et al., 2013). Similarly, Srivastava et al. (2011) reported that the viability profile showed that necrosis of cells occurs due to aggregation and clumping at low speed (60 rpm) whereas at high speed (240 rpm) it resulted in cells ruptured.

The study on palm oil suspension culture showed more than 200% increment of biomass obtained from initial inoculum at high agitation rates (120 and 225 rpm) in bioreactor system (Choi et al., 2008). This showed that shaking conditions might enhance biomass accumulation in certain species, but over shaking resulted in inhibition of cell growth due to cell injury even though an increase in oxygen supply could be achieved through intense shaking.

In terms of compound production, agitation speeds significantly affect the ZER production. The cells grown under continuous shaking condition of 70 and 100 rpm were able to produce more than 3.60 mg/L ZER compared to cells agitated at 40 rpm (Figure 3.12b). This might be due to the increase in cells stress condition caused by higher agitation speed.

In contrast for *Boesenbergia rotunda* cell suspension culture, Yusof et al. (2013) reported that total flavonoids were not significantly influenced by the agitation speed used. However, production of some individual flavonoids, such as pinostrobin and panduratin A displayed contrasting results (Yusof et al., 2013). This concluded that aeration and agitation were essential variables influencing the growth and viability of cells as well as compound accumulation.

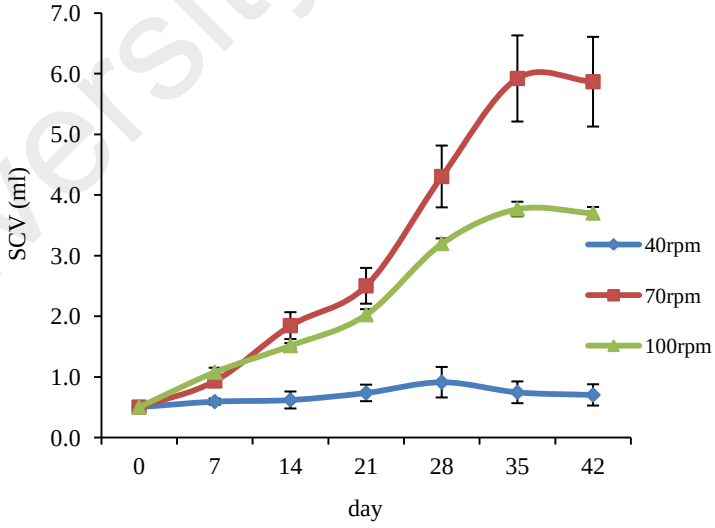

Figure 3.12a: Cell growth in different agitation.

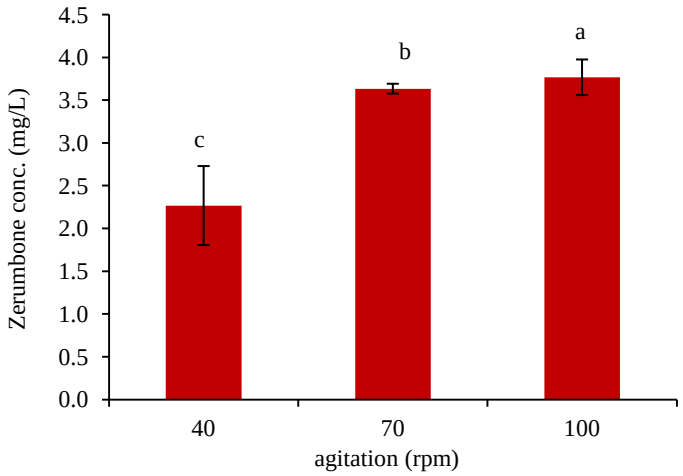

Figure 3.12b: Zerumbone production in different agitation.

3.3.5.6 Effect of pH on biomass growth and zerumbone production

Initial pH of medium prior to autoclaving was also investigated. Medium adjusted to pH5.7 recorded the highest specific growth rate of 0.0707 (Figure 3.13a). Increased in pH of medium from 5.7 to 6.2 was found to decrease the cells multiplication. As for ZER production, the best recorded concentration of ZER was in media with initial pH 5.2 (4.00 ± 0.26 mg/L). In general, pH tested in this study did not affect the production of ZER (Figure 3.13b). The pH was usually adjusted to between 5 and 6 before autoclaving to avoid extreme pH. It was also due to the need of preventing pH changes during the culture period by achieving an equilibration of medium pH before explants inoculation (Mo & Williams, 1997).

Another study revealed that medium pH significantly reduced shoot height in tomato cv. red coat but callus production and growth remained unaffected (Bhatia & Ashwath, 2005). This suggested that the explants of tomato cv. Red Coat were moderately tolerant to a broad range of pH since most of the traits investigated remained unaffected by the alteration in medium pH (Bhatia & Ashwath, 2005). However, the most excellent regeneration and growth occurred within the range of pH 5.5-6.0. This fact was further

supported by a study on the induction of friable callus from leaf explants of *Aquilaria malaccensis* for the purpose of establishing cell suspension culture, which was highly effective at pH 5.7 (Jayaraman et al., 2014).

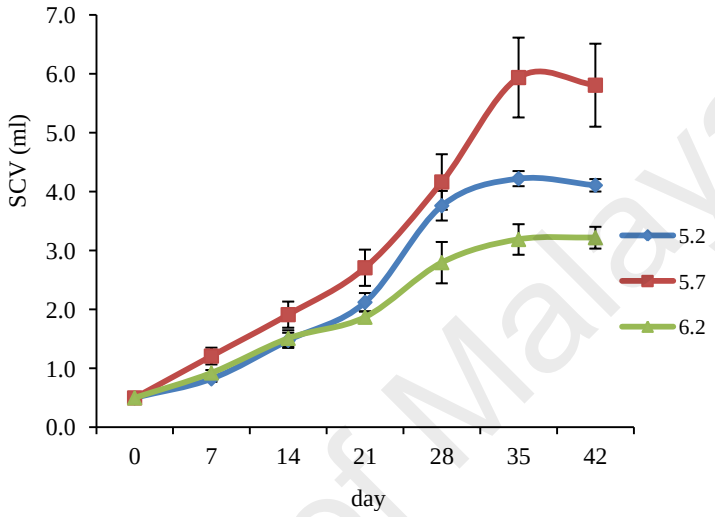

Figure 3.13a: Cell growth in different initial pH.

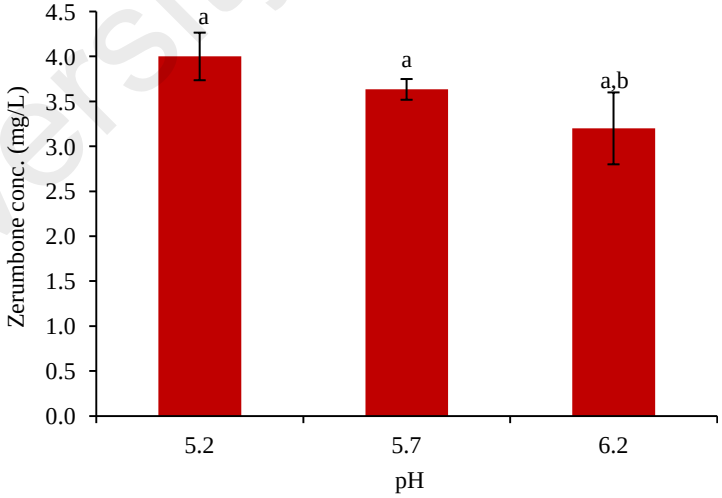

Figure 3.13b: Zerumbone production in different initial pH.

3.3.6 Comparison between zerumbone concentration in rhizome and cell suspension cultures.

The production of secondary metabolites from cell suspension has been widely investigated in various types of plants. In this study, the comparison of ZER accumulation in rhizome and cell suspension cultures of *Z. zerumbet*, in different medium and cultural conditions have been made. In this work, rhizome was found to produce abundant ZER compared to cell suspension culture. Generally it can be concluded that medium with different composition was found to affect the growth of *Z. zerumbet* cell suspension culture as well as the production of ZER. Production of ZER in *Z. zerumbet* cell suspension culture might be influenced by the viability of cells and culture conditions. Endress (1994) stated that synthesis of secondary compound has been linked to a degree of differentiation of individual cells while de Klerk (2004) stated that the lack of tissues and organ differentiations in cell cultures may cause low yield of secondary metabolites.

It is also worth noting through this work that ZER was found secreted into spent liquid medium and only some residue of ZER detected in the biomass of *Z. zerumbet* cell suspension cultures which could be due to cell lyses. Although ZER produced was not as abundant compared to rhizome, but the possibility of producing ZER during early stage could serve as a model to improve secondary compound metabolites using cell cultures. In working with cell suspension cultures system, it is crucial to increase and accumulate maximum active biomass in order to achieve the higher possible production of bioactive compound. This is because compound production from undifferentiated cells is only feasible through enough biomass. Due to its pharmaceutical and medicinal value, ZER has a great prospect to be explored for large scale production. Therefore in the next chapter, attempt in initiating adventitious root culture for ZER production will be discussed.

CHAPTER 4: ESTABLISHMENT OF ADVENTITIOUS ROOT CULTURES AND THE EFFECT OF DIFFERENT CULTURE CONDITIONS AND MEDIUM ON THE BIOMASS AND ZERUMBONE PRODUCTION

4.1 INTRODUCTION

4.1.1 Adventitious root cultures

Plant roots serve as a source of bioactive molecules that include a diversity of metabolites, proteins, agrochemicals, flavors, dyes and fragrances (Bais et al., 2001; Fulzele et al., 2002). Through the advancement in plant biotechnology, the organ culture, in particular adventitious root culture, has been applied in many medicinal plants due to its rapid growth and stable secondary metabolites production of pharmaceutical and nutraceutical importance (Ramachandra & Ravishankar 2002; Verpoorte et al., 2002; Murthy et al., 2008).

In recent years, there is a great interest in commercial production of important secondary metabolites, particularly in secondary metabolism and possibility to alter the bioactive plant metabolite production by means of cell technology. As compared to other culture systems (cell, embryo, and intact plant), root culture (hairy and adventitious) is widely exploited for the production of bioactive compounds due to high efficiency and similarities with those from mother plants (Cui et al., 2010a). Among root culture systems, adventitious root culture (AdR) is the most attractive system for the production of biomass and commercially important metabolites for the improvement and enhancement of plant-based pharmaceutical compounds (Wang et al., 2013).

In addition, AdR are the potential biological material under *in vitro* conditions for stable production of commercially valuable secondary metabolites without the presence of a foreign gene. The *in vitro* AdR culture serves as an attractive option as it is highly differentiated and be able to provide stable and extensive production of secondary

metabolites (Wadegaonkar et al., 2006; Verma et al., 2002). Furthermore, beside stable secondary metabolite production, AdR culture encompasses a remarkable characteristic for a successful scale-up such as high biomass growth, sensitivity to external stimuli and in some cases release of metabolite into the culture medium (Baque et al., 2013; Ahmad et al., 2015).

Naturally, biotic and abiotic stress conditions alter the synthesis of bioactive compounds in plants. Therefore, the synthesis of bioactive compounds in adventitious root culture can be altered by exposure to different conditions. In addition, it is relatively easy to control the physical and chemical environmental conditions of AdR cultures (Sivanandhan et al., 2012). Induction of AdR in phytohormone-supplemented medium under sterile condition have revealed high proliferation rate, tremendous potentialities of compound accumulation and stable production of valuable secondary metabolites (Hahn et al., 2003; Yu et al., 2005b). AdR formation is a complex process involving various endogenous and exogenous factors and the development is basically based on anatomical basis. Compared to cell cultures, AdR displayed higher stability in their growing environment and synthesize enormous amounts of secondary metabolites into their intercellular spaces, which can be more easily extracted (Cui et al., 2010a; Baque et al., 2013). AdR can be cultured in a phytohormone-amended medium with low inoculum yet displayed a high growth rate (Sivakumar, 2006).

It is well known that the development of root is a complex trait controlled by hormonal signals, particularly auxins and that the environmental cues can operate the genetic programs for root development and their hormonal and metabolic control (Zhang et al.,1999). Sorin et al. (2005) further mentioned that AdR formation is an intricate process due to the involvement of multiple endogenous factors and

environment aspects such as light and wounding. The process of differentiation and induction pathways in rooting can be triggered by exogenous supplementation of auxins (Praveen et al., 2009b). The physiological stages of rooting correlate with the changes in endogenous auxin concentrations (Heloir *et at*., 1996) and the rhizogenic role of auxins has been considered most essential for initiation and establishment of adventitious roots (Weisman et al., 1988).

4.1.2 Bioactive compounds in adventitious roots

Chemical synthesis of bioactive compounds is a complicated and expensive (Shimomura et al., 1997). While *in vivo* production of these compounds is influenced by several factors such as cultivation period, collection season, plant-to-plant variability, adulteration of medicinal preparations with misidentified plant species and lack of satisfactory methods for crop production and standardization. In the search for an alternative solution to the problem faced by the phyto-pharmaceutical industries, biotechnological approaches, specifically plant cell culture, has been found to have great potential to enhance conventional agriculture for the industrial production of bioactive plant metabolites (Ramachandra & Ravishankar, 2002). Furthermore, the rapid production of the plants as well as the induction of *in vitro* roots is the most feasible strategy to get rid of the stress on the natural population.

The advancement of plant root cultures is capable of producing particular medicinal compounds at a similar rate or greater to that of naturally grown plants (Baque et al., 2012a). Over the years, the number of bioactive compounds extracted from AdR culture is increasing. A list of pharmaceutically important medicinal plants, in which AdR have been induced and cultured successfully for the efficient production of high value secondary metabolites, is depicted in Table 4.1.

Even though the success of AdR cultures has been well documented in many plant species, there are only a few bioactive compounds successfully commercialized. This is because the purifying of the compound is often difficult and expensive from such cultures. Therefore, in order to exploit the AdR for large-scale commercial production of useful bioactive compounds, the knowledge on the regulation of secondary metabolic pathways concerned with the level of products, enzymes and genes, as well as transport and compartmentation are required.

Table 4.1: List of plants which adventitious roots have been induced and cultured successfully for the production of highly-valued secondary metabolites.

Plant species	Metabolites	Importance	References
Panax ginseng	Ginsenosides	Anticancer, antifatigue, immunostimulant, anti-inflammatory, antioxidant	Paek et al., 2009; Son et al., 1999; Choi et al., 2000; Kim et al., 2005; Jeong et al., 2006
Panax notoginseng	Saponin	Immunostimulant, anticancer	Gao et al., 2005
Morinda citrifolia	Antraquinone, rubiadin, phenolics, flavonoids	Anticancer, antialergic, antibacterial, antiviral, hepatoprotective, antioxidant	Baque et al., 2010a, 2010b, 2010c; Baque et al., 2011
Echinacea purpurea *E. angustifolia*	Caffeic acid derivatives	Immunostimulant, anti-inflammatory	Wu et al., 2006, 2007; Jeong et al., 2009
Hypericum perforatum	Hypericin, hyperin	Antidepressive, antifungal, anti-inflammatory	Cui et al., 2010a, 2010b, 2010c; Cui, 2011
Eleutherococcus koreanum	Eleutheroside B,E Chlorogenic acid	Antifatigue, analgesic	Lee et al., 2011
Pelargonium sidoides	Coumarin	Antivirus, antibiotic	Jeong, 2009
Scopolia parviflora	Hyacyamine (alkaloid)	Anticholinergic activity	Kang et al., 2004
Rapanus sativus	Anthocyanin	Food colouring	Betsui et al., 2004
Rhus javanica	Galloylglucoses, reccionidin A	Antioxidant	Taniguchi et al., 2000
Dubosia myoporoides *D. leichhardtii*	Scopolamine	Spamolytic, kydriatic agents	Yoshimatsu et al., 2004
Cornus capitata	Tannins	Antioxidants	Tanaka et al., 2001
Anthemis nobilis	Geranyl isovalerate	Anti-inflammatory, fragrance, essential oil	Omato et al., 1998
Andrographis paniculata	Andrographolide	Antimalarial, antipyretic	Praveen et al., 2009b

Source: Baque *et al*. (2012)

4.2 MATERIALS AND METHODS

4.2.1 Plant material

The rhizome of *Zingiber zerumbet* Smith used for this experiment was obtained from the same source according to section 3.2.1. The method to induce sprouted buds was according to procedure in section 3.2.1, Chapter 3.

4.2.2 Sterilization procedure

4.2.2.1 Explant sterilization

Explant sterilization was done only for AdR initiated through direct pathway. The sterilization was done according to procedure in section 3.2.2.1, Chapter 3 while for AdR initiated through indirect pathway; sterilization was not needed.

4.2.3 Initiation of adventitious root cultures

4.2.3.1 Type of inoculum

The AdR was initiated through two different pathways, direct and indirect (through callus stage). The AdR initiated through direct pathway is known as AdRD while the one initiated through indirect pathway is AdRId. The AdRD was initiated by culturing slices of shoot buds induced in 4.2.1 while AdRId was initiated by culturing friable calli onto initiation media. The callus was initiated following the procedure in 3.2.3.1, Chapter 3.

4.2.3.2 Plant growth regulators (PGR)

Two types of auxins, indole-3-butyric acid (IBA) and 1-naphthaleneaceatic acid, (NAA) acquired from Sigma (USA) were used in the range of 0.5, 1, 2, 3, 5, 7, 9 mg/l for initiation of AdR culture. Media without PGR served as control. The induction frequency, number and length of AdR formed were recorded after 2 months of

initiation. All experiments were carried out in triplicate cultures with five explants per replicate and each treatment was repeated twice.

4.2.3.3 Culture condition

The media used for initiating AdRD and AdRId were Murashige and Skoog (MS) basal medium (Appendix 1) supplemented with 3% sucrose, two different types of auxin IBA and NAA at different concentrations and solidified with 2 g/l phytagel. The media pH was adjusted to 5.7 prior to autoclaving. All cultures were maintained at 25 ± 1^0C under a dark photoperiod.

4.2.4 Initiation of adventitious root suspension cultures

4.2.4.1 Inoculum type

Initiation of AdR suspension cultures was done by using two different types of inoculums namely roots initiated from indirect pathway (AdRId) and direct pathway (AdRD). Both were initiated according to procedure in 4.2.3, Chapter 4.

4.2.4.2 Plant growth regulators (PGR)

For both AdRId and AdRD, 0.5 g of inoculum was transferred to liquid media MS medium supplemented with 3% sucrose, different concentrations of IBA and NAA (1, 2, 3, 5, 7, 9 mg/l). All treatments were replicated trice and the experiment was repeated three times.

4.2.4.3 Light regime

The effect of light regime was investigated by incubating the AdR suspension cultures in two conditions, 16 h light : 8 h dark and complete dark. Light was provided by fluorescent lamp with light intensity of 31.4 µmol $m^{-2}s^{-1}$ and cultures were placed in

boxes to simulate dark condition. All cultures were incubated at 25 ± 2^0C in the growth room.

4.2.4.4 Determination of cell biomass

Root biomass was harvested after 2 months of culture to determine fresh weight (FW) and dry weight (DW). The fresh root biomass was collected from liquid media and carefully washed with distilled water and pressed gently on filter paper (Whatman Ltd., England) to remove excess water and finally weighed (A&D GF 300 digital balance, Japan). Similarly, for dry biomass (DB) determination, roots were dried in an oven (Memmert, Germany) at 38°C for 48 h and finally weighed. All treatments were replicated trice and the experiment was repeated three times.

4.2.4.5 Histological section of roots

Both AdRD and AdRId specimens (two months old) selected for histological studies were fixed for 24 h in formaldehyde-absolute ethanol-acetic acid (FAA) solution consisting of 5% (v/v) formaldehyde, 45% (v/v) absolute ethanol and 5% (v/v) glacial acetic acid. The samples were then dehydrated in an ethanol series; 30% for 30 min, 50% for 45 min, 70% for 45 min, 80% for 60 min and 100% for 120 min. Specimen were then embedded in basic resin (Technovit 7100, Germany) and sectioned at 3.5 μm. Sections were double-stained with naphtol blue black (Sigma, USA) [1 g naphthol blue black in 100 ml 7% (v/v) acetic acid] and periodic acid [1% (w/v)](Fisher, 1968).

4.2.5 Multiplication and growth of adventitious root suspension culture

4.2.5.1 Type of inoculum

Two types of inoculum namely AdRId and AdRD were used in optimizing growth and multiplication of AdR suspension cultures. Both inoculums were prepared according to procedure in section 4.2.3.1, Chapter 4.

4.2.5.2 Culture conditions

The multiplication of roots was done on the optimized media obtained from the previous experiment in section 4.2.3, Chapter 4. All cultures were incubated at 25 ± 2^0C in 16 h light : 8 h dark light regime with light intensity of 31.4 μmol $m^{-2}s^{-1}$. All treatments are subjected to 3% of sucrose and all media were adjusted to pH 5.7 prior autoclaving and culture under continuous shaking condition at 80 rpm. All experiments were carried out in triplicate cultures and each treatment was repeated three times.

4.2.5.3 Initial inoculum density

The effects of initial inoculum density were observed at different weight of roots (0.5, 1.0, 2.0, 3.0 and 5.0 g) for both AdRId and AdRD. The AdR were weighed and inoculated into 50 ml fresh liquid media. All experiments were carried out in triplicate cultures and each treatment was repeated three times.

4.2.5.4 Medium salt strength

The effects of MS basal medium strength on biomass accumulation and ZER production from *Z. zerumbet* adventitious root culture were also examined. The inoculum volume used was 0.5 g. The strengths of MS basal medium were halved, singled and doubled the concentrations prepared following standard formulation (Appendix A). All experiments were carried out in triplicate cultures and each treatment was repeated three times.

4.2.5.5 The growth profile of adventitious root suspension cultures

The growth of AdR suspension cultures was measured by weighing the fresh weight (FW) every 5 days for a period of 30 days whereas the specific growth rate (the changes of SCV in natural log) was recorded for 25 days of culture. All experiments were carried out in triplicate cultures and each treatment was repeated three times.

4.2.6 Preparation of extract for analysis of zerumbone

4.2.6.1 Rhizome

The extraction of rhizome was done according to procedure in section 3.2.6.1, Chapter 3.

4.2.6.2 Adventitious root culture

Extraction of ZER was done on AdR suspension culture during initiation and multiplication stage. The optimization of ZER production was done by using AdR suspension cultures initiated from AdRId and AdRD cultured in MS media with 3% sucrose supplemented with different concentrations of IBA and NAA subjected to light and dark regime as stated in 4.2.4.2 and 4.2.4.3. Extraction of zerumbone was done for both types of AdR suspension cultures subjected to different inoculum volume and MS salt strength. Roots for all treatments were harvested at day 25. All biomass were extracted by using Soxhlet to determine the presence of zerumbone.

4.2.7 Identification of zerumbone compound

The identification of zerumbone compound was done according to procedure in section 3.2.9, Chapter 3.

4.2.8 Extraction and HPLC analysis

Harvested root biomass was dried at 38°C until constant weight was achieved prior to grinding using mortar and pestle. 0.5 g of powder was extracted for ZER and analyzed as described in section 3.2.9, Chapter 3.

4.2.9 Statistical analysis

All data collected were analyzed by one way ANOVA followed by Duncan test at a significance level of $p < 0.05$. Replicates are indicated in each section.

4.3 RESULTS AND DISCUSSION

4.3.1 Initiation of adventitious root culture

4.3.1.1 Effect of plant growth regulator on adventitious root induction

Adventitious roots (AdR) were succesfully initiated through direct and indirect pathways. Both pathways produced AdR with a slight difference in morphology and time of appearence. AdR initiated through indirect pathway (AdRId) produced numerous roots with shorter and fine structure (Figure 4.1c) compared to AdR initiated from direct pathway (AdRD) (Figure 4.2b).

For AdRD, slices of shoot buds induce roots as early as 1 week of culture whereas AdRId emerged from the calli within 2 weeks of culture. This was similar to the initial visibility of adventitious roots of *Gynura procumbens* which appeared after 7 days of inoculation (Saiman et al., 2012) and indirect rhizogenesis of *Castilleja tenuiflora* Benth was also observed around the surface of leaf explants after 1 week of culture (Gómez-Aguirre et al., 2012). Whereas in the case of *Plumbago zeylanica* L. adventitious root culture, the explants cultivated on auxins supplemented medium

induced roots from the cut ends and veins within 2 weeks incubation (Sivanesan & Jeong, 2009).

For AdRId, explants produced callus after 2 weeks of incubation (Fig. 4.1A) and later multiplied into friable callus (Figure 4.1B) within 1-2 months of culture on callus initiation media (Jalil et al., 2015). Upon transferring onto root initiation media, numerous short and tiny roots emerged from the friable callus (Figure 4.1C).

As for AdRD, roots emerged directly from the shoot bud explants (Figure 4.2A) and produced a few roots within 2 weeks of culture (Figure 4.2B). After 2 months of incubation on the same media, AdRD produced masses of roots (Figure 4.2C) which were ready to be multiplied in liquid media. The histological section on both roots showed that the cells in AdRD typified normal root anatomy with the presence of apical meristem and procambium whereas in AdRId only apical meristem existed (Figure 4.1D and 4.2D). Nonetheless, both roots possessed root caps as found in normal roots.

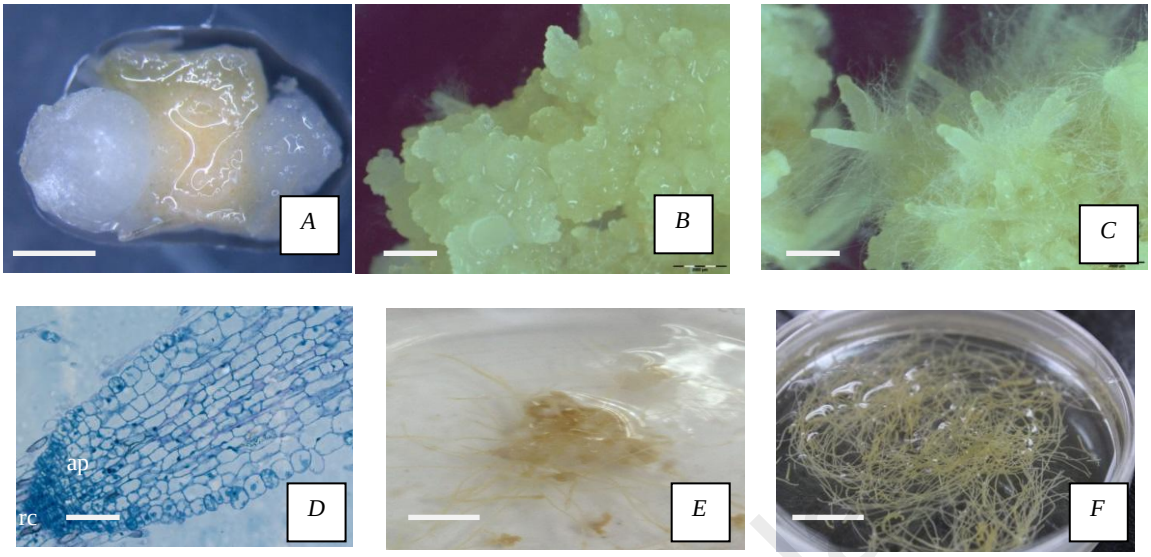

Figure 4.1: Initiation of adventitious root from indirect pathway (AdRId); *A* : callus initiated from shoot bud slice explant. *Bar : 1 mm*; *B* : callus multiplication. *Bar : 1 mm*; *C* : initiation of adventitious root (AdRId). *Bar : 2 cm*; *D* : histology of AdRId. *Bar : 100 µm*; *E* : initiation of AdRId suspension culture. *Bar : 2 cm*; *F* : multiplication of AdRId. *Bar : 2 cm*. *rc* : root cap; *ap* : apical meristem.

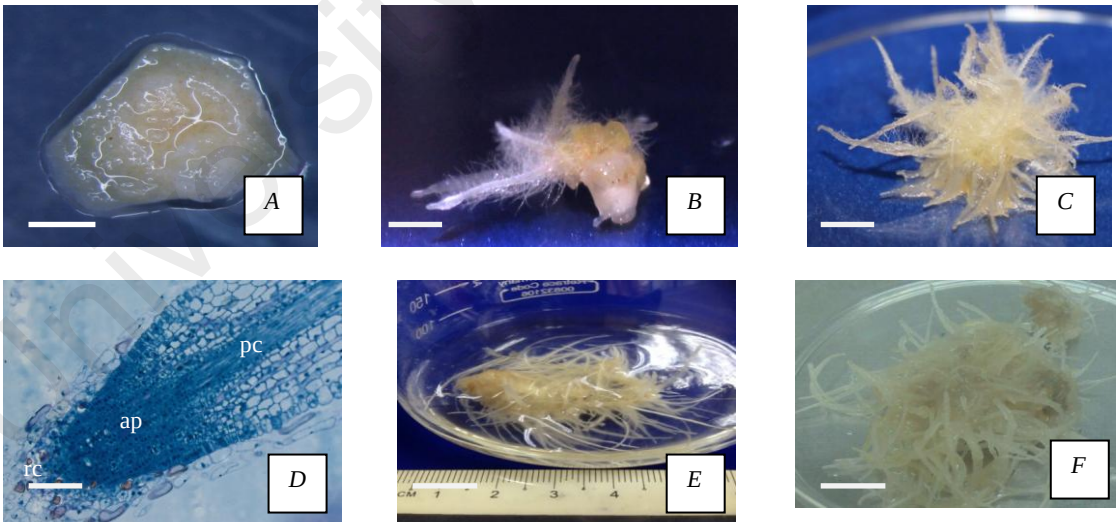

Figure 4.2: Initiation of adventitious root from direct pathway (AdRD); *A* : shoot bud slice explant. *Bar : 1 mm*; *B* : initiation of AdRD. *Bar : 1 cm*; *C* : elongation of AdRD. *Bar : 2 cm*; *D* : histology of AdRD. *Bar : 100 µm*; *E* : AdRD suspension culture. *Bar : 2 cm*; *F* : multiplication of AdRD. *Bar : 2 cm*. *rc* : root cap; *ap* : apical meristem; *pc* : procambium.

For initiation frequency, the percentage of AdR formation was higher through indirect pathway in which AdRId showed 100% initiation in MS medium supplemented with IBA (2 mg/L and 3 mg/L) and MS media supplemented with 3 mg/L NAA (Fig. 4.3b). While for direct pathway, the highest percentage (88%) of AdRD was obtained in 2 mg/L IBA (Fig. 4.3a). The formation of AdR was best at lower concentration of plant growth regulator for both IBA and NAA. Higher auxin concentration (5-9 mg/L) showed an inhibition pattern in AdR formation for both direct and indirect pathways. Lowering the auxin concentration to 0.5 mg/L also did not support the AdR initiation which might due to insufficient supply of hormone to trigger the cell divisions and root primodia organization.

In our finding, IBA was significantly preferred compared to NAA in *Z. zerumbet* adventitious root formation. This was in accordance with findings by Baque et al. (2010b) who found that IBA was more effective than NAA in inducing adventitious roots from leaf segments of *Morinda citrifolia*. Similar results were also obtained in *Podophyllum peltatum* (Anbazhagan et al., 2008), *Echinacea angustifolia* (Wu et al., 2006) and *Raphanus sativus* (cv. Peking Koushin) root culture (Betsui et al., 2004). In contrast, NAA was favorable for inducing adventitious roots from leaf explants of *Andrographis paniculata* (Praveen et al., 2009b), *Castilleja tenuiflora* Benth (Gómez-Aguirre *et al*., 2012) and *Gynura procumbens* (Saiman et al., 2012). Jenifer et al. (2012) had found that NAA was more potent in triggering AdR from the *Boerhaavia diffusa* leaf explant and similar results were also observed in *Robinia psuedoacacia* where NAA was found to induce rooting (Swamy, 2001). Nevertheless, synergistic effect of NAA and IBA was also proven to enhance the regeneration of adventitious roots. In *Psammosilene tunicoides* adventitious root culture, the combination of NAA and IBA significantly affect the adventitious roots formation (Zhang et al., 2013). This suggests

that the response of root organogenesis to different auxins varies based on different pathway and plant species.

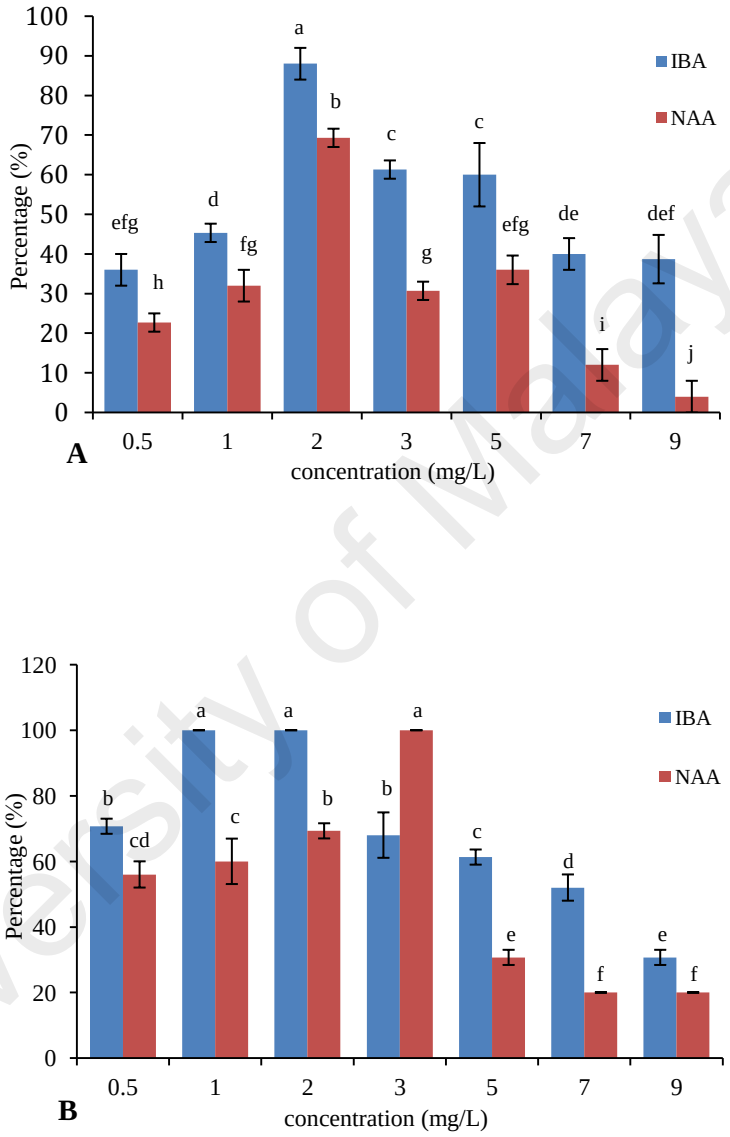

Figure 4.3: Percentage of AdR initiated through direct and indirect pathway in different concentrations of auxin. *A* : direct pathway; *B* : indirect pathway.

In contrast to initiation frequency of root, the amount of AdR produced for both direct and indirect pathways were significantly higher in NAA supplemented media. The indirect pathway produced higher amount of AdR compared to direct pathway. For indirect pathway**,** the highest number of AdRId per explant was achieved in media supplemented with 3 mg/L NAA (19 AdR/explant) whereas 7.7 AdR/explant was obtained in media containing 1 mg/L IBA (Figure 4.4b). As for direct pathway, the highest number of AdR per explant was in media supplemented with 5 mg/L NAA (16.6AdR/explant) while for media supplemented with IBA, the highest number of AdRD was achieved in media with 5 mg/L IBA (11 AdR/explant) (Figure 4.4a). This showed that AdRId preferred lower concentration of auxins compared to AdRD which may be due to the presence of endogenous auxin in callus based explant as auxin is crucial for root induction (Takahashi, 2013). In *Plumbago zeylanica* adventitious roots culture, the highest number of roots was obtained in MS medium with a combination of auxin (1.0 mg/L IBA and 0.5 mg/L NAA) which produced 19 roots/explant (Sivanesan & Jeong, 2009).

The amount of AdR produced also require a moderate concentration of auxin in which lowering its concentration (0.5-2 mg/L) or increasing it more than 5mg/L resulted in low number of AdR. Similar result was achieved in *Gynura procumbens* root culture, in which increasing NAA concentration resulted in high biomass accumulation with the significant NAA concentration at 5mg/L NAA but it was in contrary with IBA supplemented media in which there was no escalation of biomass with the increasing of IBA concentration (Saiman et al., 2012). While Baque et al. (2012a) observed total absentees in the formations of adventitious roots in culture containing concentration of 5 mg/L NAA.

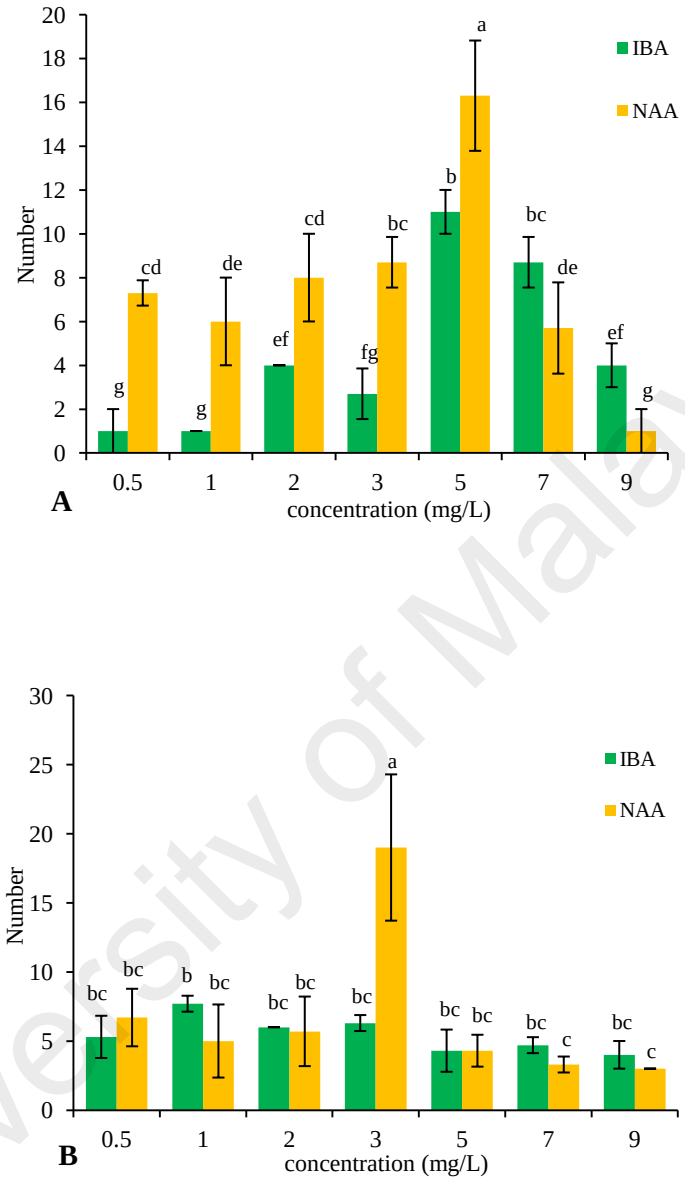

Figure 4.4: Number of AdR initiated through direct and indirect pathways in different concentrations of auxin. *A* : direct pathway; *B* : indirect pathway.

Root elongation resulted from the combined effects of mitotic activity of root cells and the enlargement of cellular components in the direction of the longitudinal axis (Overvoorde et al., 2010). In contrast to amount of root produced, the root elongation was significantly superior in IBA supplemented media in which AdRD produced longer roots compared to AdRId. For both AdRId and AdRD, the optimum medium for root length was media supplemented with 5.0 mg/L IBA. The length of AdRD in media supplemented with IBA and NAA ranging from 5.5-8 cm and 1.6-6.3 cm respectively after two months in culture. Maximum length of AdRD obtained was 8 cm and 6.3 cm in media with 5.0 mg/L IBA and 2.0 mg/L NAA respectively (Figure 4.5A).

As for indirect pathway, the length of AdRId in media supplemented with IBA and NAA ranging from 2.5-6.0 cm and 1.0-3.5cm respectively after two months of culture. Maximum length of AdRId obtained was 6.0 cm and 3.5cm in media with 5.0 mg/L IBA and 2.0 and 3.0 mg/L NAA respectively (Figure 4.5B). This was in accordance with the finding in the adventitious roots culture of *Eurycoma longifolia*. The AdR of *Eurycoma longifolia* cultured in 5.0 and 7.0 mg/L IBA were numerous (>10) and more than 1.0 cm in length, in comparison to roots cultured in 5.0 mg/ L NAA which were thicker, less numerous and shorter (Lulu et al., 2015). This may be due to the nature of IBA as an endogenous auxin and the influence of IBA as a source of auxin over a period of time or that IBA conjugates are a better source of free auxin through hydrolysis (Yang & Davies, 1999). It was further supported by this study where AdRD seemed to contain more meristematic cells compared to AdRId which might contribute to longer root formation (Figure 4.1D and 4.2D).

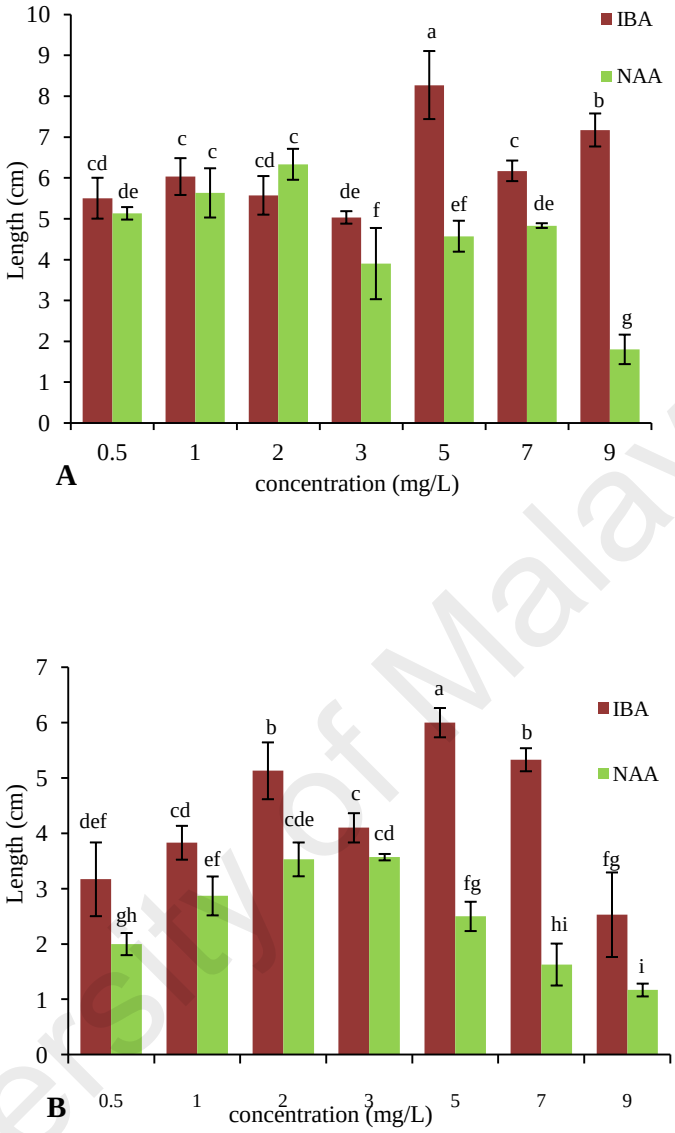

Figure 4.5: Length of AdR initiated through direct and indirect pathways in different concentration of auxins. *A* : direct pathway; *B* : indirect pathway.

4.3.2 Establishment and multiplication of adventitious root suspension cultures

4.3.2.1 Effect of plant growth regulator on adventitious root multiplication and zerumbone production

In order to explore future potential for a large-scale production in bioreactor, the AdR obtained from both direct and indirect pathways were proliferated in liquid media for further optimization. Auxins are required for the maintenance of plant cell and tissue culture systems, wherein they are associated with the promotion of growth, rooting, callus proliferation, and also offer morphological diversity (Kim et al., 2004a; Lee et al., 2006). Thus, in order to multiply *Z. zerumbet* AdR culture, the effect of auxin on biomass and compound accumulation were investigated. Depending on the concentration used, auxin treatment was reported to either stimulated or inhibited root production (Overvoorde et al., 2010).

In our study, both types of inoculum (AdRD and AdRId) responded in liquid medium producing masses of AdR suspension cultures (Figure 4.1E and Figure 4.2E). After 2 months of culture, AdR suspension cultures initiated from AdRId produced highest biomass, 39.214 g fresh weight (FW) and 4.414 g dry weight (DW) in MS media supplemented with 3 mg/L NAA incubated in 16:8 h/day light regime (Figure 4.6A). However, the highest zerumbone production was obtained from AdRId suspension cultured in dark condition with 7 mg/L IBA (Figure 4.6B) but showed lower biomass accumulation (3.462 g FW). The HPLC profile was shown in Appendix D. For AdRD suspension culture, the optimum medium for biomass production was MS supplemented with 1mg/L NAA (dark condition) with the accumulation of biomass of 9.739 g FW (1.121 g DW) after 2 months of culture (Figure 4.7A).

Nevertheless it was low in zerumbone production (606 μg/g DW). For AdRD, ZER was highest in media supplemented with 7 mg/L IBA and NAA in 16h/d light regime that is 1016 and 1826 μg/g DW respectively. The HPLC profile was shown in Appendix E. The role of NAA in the enhancement of AdR was well documented. Hussain *et al*. (2012) found that application of NAA is the best option to produce adventitious rooting in *Eurycoma longifolia* whereby, Lee et al. (2011) reported that lower concentration of NAA accelerated AdR production in *Aloe vera*.

From this study, increasing the auxins concentration triggered higher ZER compound but suppress markedly the biomass accumulation (Figure 4.7B). This could be due to the fact that at high concentration, auxins may possess herbicidal property thus hampering the root formation (Sterlling & Namuth, 2004). The above finding was in contrast with Lulu et al. (2015), who stated that the *Eurycoma longifolia* root culture produce higher FW and DW of roots with the increasing NAA concentration up to 5 mg/L but the development of *E. longifolia* AdR was not inhibited in medium supplemented with 7.0 or 9.0 mg/L NAA. While the bioactive compound accumulation pattern was similar as in *Z. zerumbet* root culture. The yield of phenolics and flavonoids of *Eurycoma longifolia* adventitious roots increased significantly with the IBA concentration with the maximum phenolic contents were 7.55 mg/g DW with 9.0 mg/L IBA and 10.30 mg/g DW with 9.0 mg/L NAA (Lulu et al., 2015). Taking into account of the biomass accumulation and ZER concentration, media supplemented with 1 mg/L NAA was selected as the optimum media for AdRD with 8.240 g FW and 806.77 μg/g ZER production which could be manipulated for further work. The induction of healthy adventitious roots with acceptable amount of ZER as the key to secondary compound accumulation is considered acceptable.

This suggests that different auxin concentrations significantly affect zerumbone production and biomass accumulation was not significantly related with ZER production. This was in accordance with Xu et al. (2002) who stated that NAA added medium supported growth of saffron callus whereas accumulation of crocin was high in IAA supplemented media.

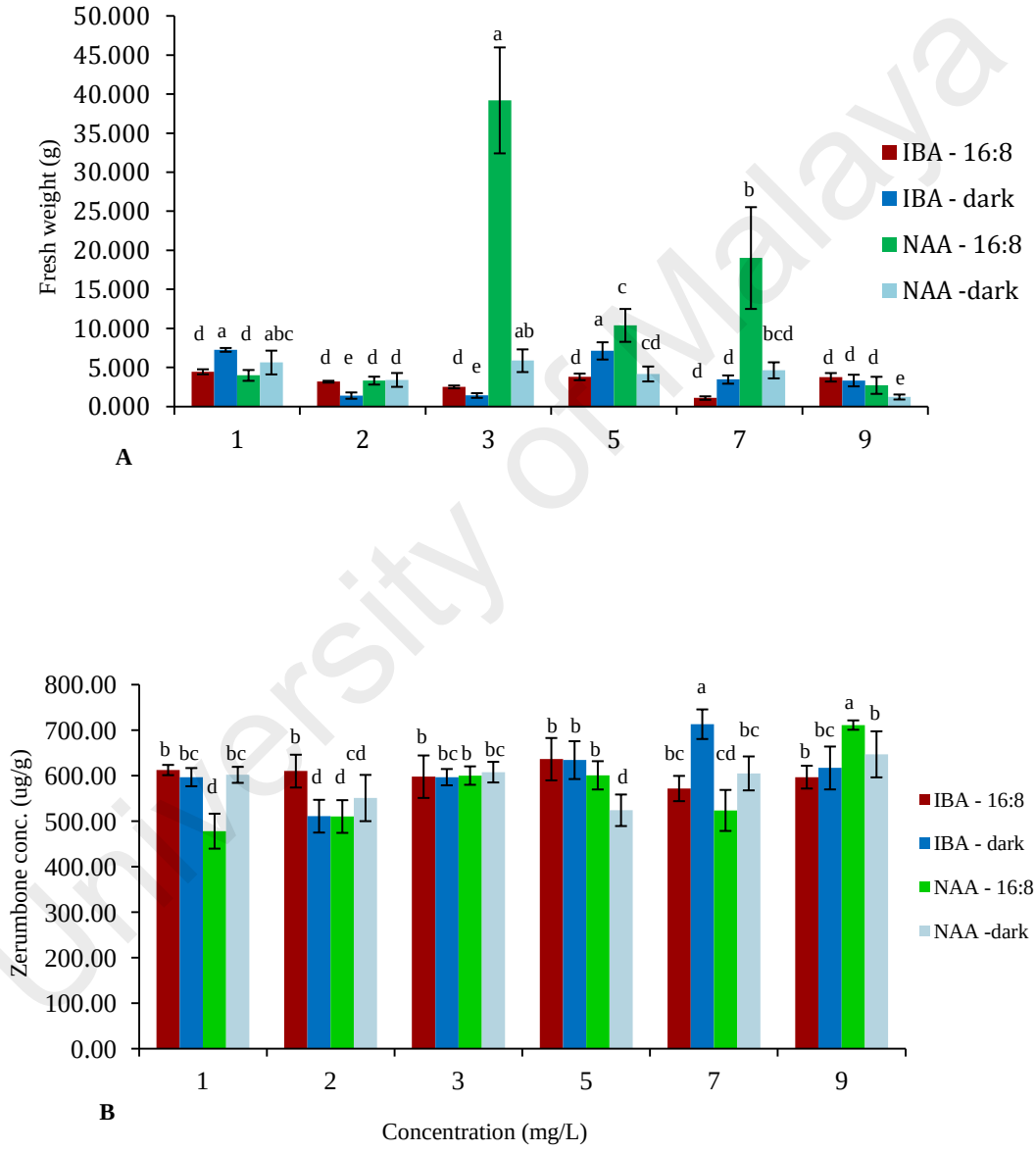

Figure 4.6: Biomass of AdR suspension culture and zerumbone production from AdRId in different concentrations of auxins and light regime. *A* : fresh biomass; *B* : zerumbone production.

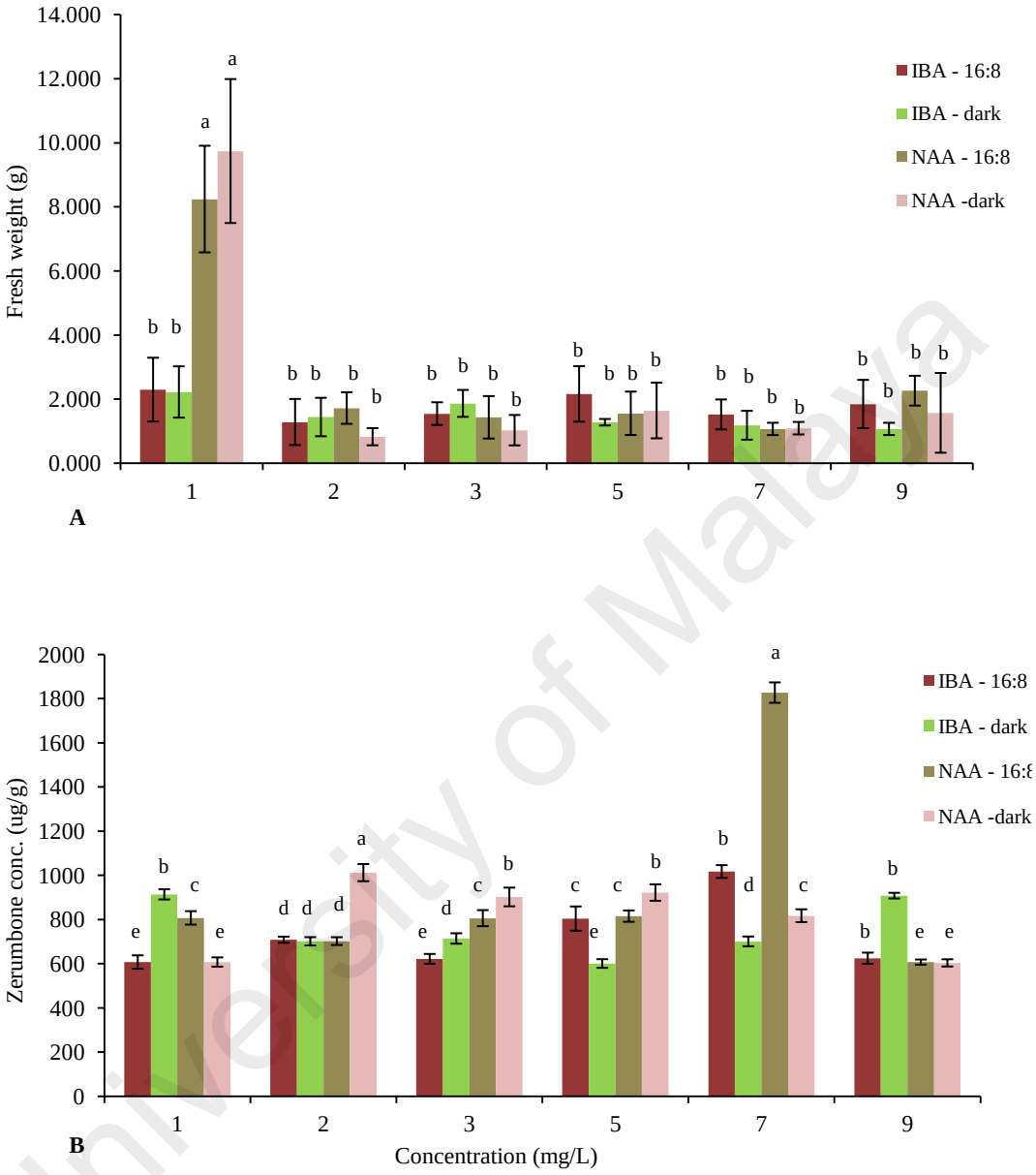

Figure 4.7: Biomass of AdR suspension culture and zerumbone production from AdRD in different concentrations of auxins and light regime. *A* : fresh biomass; *B* : zerumbone production.

4.3.2.2 Effect of initial inoculum density on biomass accumulation and zerumbone production

The multiplication of AdR suspension culture was further investigated by using initial inoculum density and medium salt strength. The initial inoculum density is one of the important parameter in determining cell growth. Previous studies shown that the secondary metabolite yield in several plant cell cultures, were found to be strongly dependent on the density or size of the inoculum at initiation stage (Lee & Shuler, 2000). From the growth profile (Figure 4.8a and 4.9a), it showed that the growth of AdR suspension cultures initiated from AdRId was superior to AdRD suspension cultures (Table 4.1). For AdRId, the maximum specific growth rate ($10.8 \times 10^{-2}\mu$) was displayed at 0.5g initial inoculum density whereas for AdRD, the maximum growth rate ($8.4 \times 10^{-2}\mu$) was observed at 1.0 g initial inoculum density (Table 4.2).

Higher inoculum density (2.0-5.0 g) resulted in lower growth ratio of AdRD which could be due to competition of nutrition which finally produced less cell multiplication. This result was similar to Cui et al. (2010a) where final weights for root biomass increased with elevated inoculum densities but the growth ratio greatly decreased in *Hypericum perforatum*. When the inoculum size was less than 6 g/l FW, low roots biomass was obtained and this was in contrast to inoculum sizes of 8 and 10 g/l FW where the biomass increased resulting in growth ratio reductions of 31.8% and 44%, respectively (Cui et al., 2010). The decrease in the growth ratio with inoculum size was perhaps due to cells competing for nutrients and oxygen at high cell densities (Zhang et al., 2002). Similar effect was also monitored in a *Catharanthus roseus* cell culture (Contin et al. 1998).

In terms of ZER production for both types of roots (AdRId and AdRD), moderate inoculum density (2.0 g) showed higher ZER compound production compared to low (0.5 and 1.0 g) and high inoculum density (5.0, 7.0 and 9.0 g) (Figure 4.8b and 4.9b). The optimum initial inoculum volume for ZER production was 2.0g initial inoculum volume for both AdRId (720 µg/g) and AdRD (983 µg/g). This was in accordance with Zhang et al. (2002), where the intracellular paclitaxel production was increased in *Taxus yunnanensis* cell suspension cultures when a moderately high inoculum size (200 g/l FW) was used.

Recent study by Yusof et al. (2013) showed that low initial inoculation volume (0.25 ml SCV) resulted in poor biomass of cells suspension of *B. rotunda* and low flavonoids yield which possibly due to inadequate critical mass of surviving cells and degree of nutrient abundance in the liquid media which negatively affect the cell growth. Zhang et al. (2002) also stated that for *Taxus yunnanensis* cell suspension cultures, the change of inoculum size had slight or no crucial effect on the specific taxol yield at most inoculum ages. Therefore, the effect of inoculum size on volumetric taxol yield and productivity was primarily a result of its effect on biomass production rather than taxol biosynthesis. Generally, inoculums size is an essential factor for cell culture propagations and could affect the accumulation of biomass and secondary metabolites production.

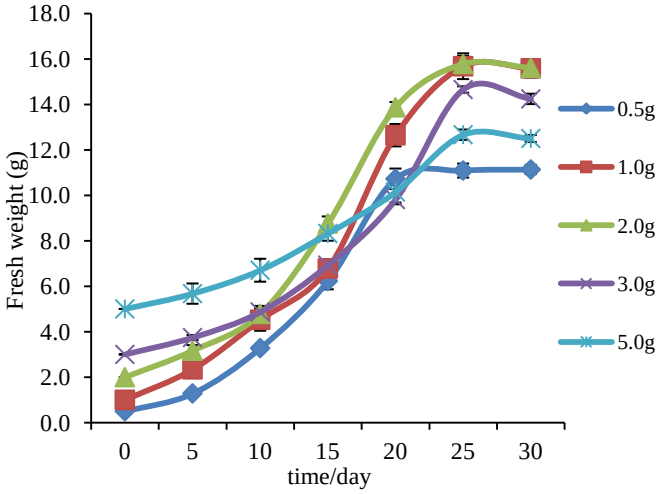

Figure 4.8a: Growth rate of AdRId in different inoculum volume.

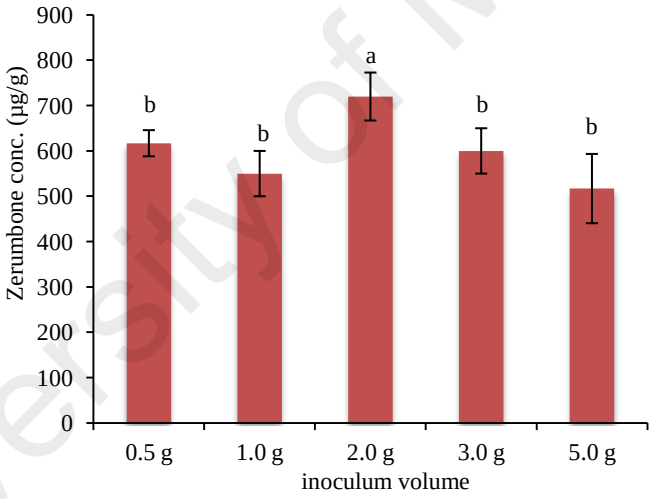

Figure 4.8b: ZER production of AdRId in different inoculum volume.

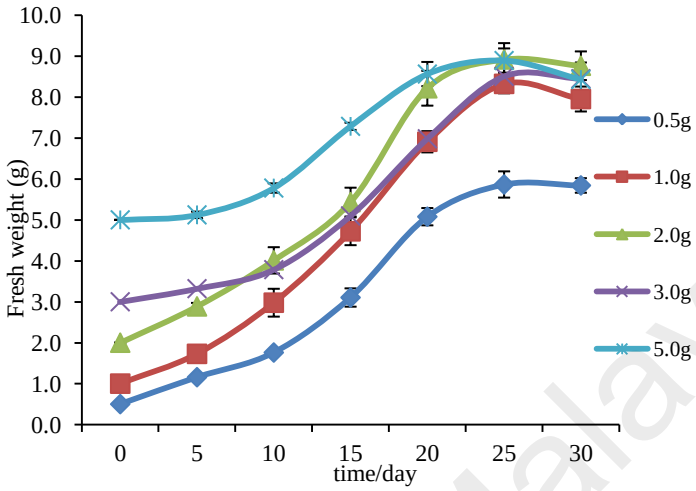

Figure 4.9a: Growth rate of AdRD in different inoculum volume.

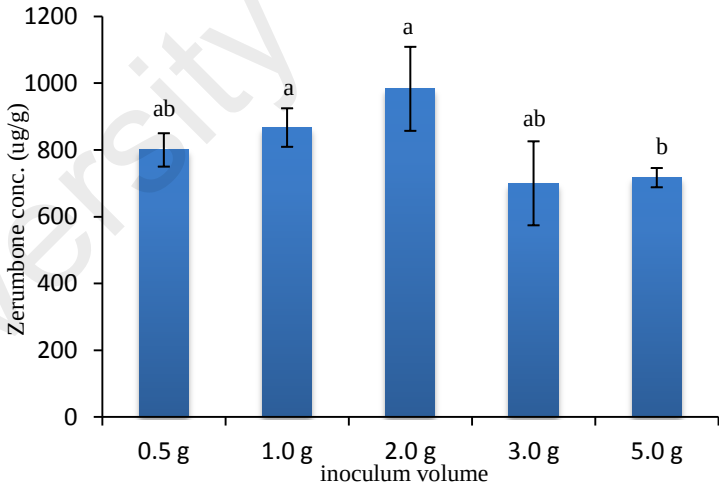

Figure 4.9b: ZER production of AdRD in different inoculum volume.

4.3.2.3 Effect of MS medium salt strength on biomass accumulation and zerumbone production

Another factor that could influence the success of *in vitro* plant culture is the composition of the medium. These media contained nutrients which is normally required by plant cells and particularly for root culture. Suitable nutrient strength was not only crucial to produce good growth of cells, but also important for secondary metabolite production (Ramachandra & Ravishankar, 2002) which have been hypothesized to be linked to the ionic balance in the medium (Drewes & Staden, 1995).

In our findings full strength MS medium favoured the biomass accumulation but the ZER production was higher in medium with half strength nutrient (Figure 4.10a and 4.10b). In both AdRId and AdRD, the maximum specific growth rate was achieved in full strength medium salt which was 11.0 and 8.4 x 10^{-2}μ respectively (Table 4.2). Nagella and Murthy (2010) found that full strength MS medium favoured the accumulation of biomass, which was in agreement with this study but contrastingly also supported the compound accumulation (withanolide A) in cell suspension cultures of *Withania somnifera*. In this study, it was also found that double strength media exhibited low root biomass but comparable ZER accumulation to half strength medium. In *Hypericum perforatum*, it was also found that doubled MS salt strengths inhibited root growth probably due to low water potential which inhibited water and mineral nutrients absorption from the medium due to osmotic stress (Cui et al., 2010a).

In half medium salt strength, low biomass production was shown but the compound accumulation in both inoculums used in this study showed maximum ZER production, that is 1500 μg/g in AdRId and 2520 μg/g in AdRD (Figure 4.10b and 4.11b). This is in

accordance to the work carried out on growth of *Eleutherococcus koreanum* Nakai where adventitious root was also better at low (1/4, 1/2, and 3/4 MS) than full and double salt strengths (Lee & Peak, 2012a). As for the total production of 5 target compounds namely eleutheroside B and E, chlorogenic acid, total phenolics, and flavonoids, it showed a decline pattern with the increasing of medium salt strength (Lee & Paek, 2012a). In *Podophyllum peltatum*, ½ MS demonstrated superlative results for adventitious root induction in comparison to full strength MS and ½ MS lacking of NH_4NO_3 (Anbazhagan et al., 2008). This might be due to insufficient nutrients at low concentration available to the cells and at high nutrient concentration an osmotic stress for the cell cultures might occur (Lipavská & Vreugdenhil, 1996). This suggests that medium strength have an effect on compound production especially in AdR culture of *Z. zerumbet*.

Table 4.2: Specific growth rate and zerumbone concentration based on inoculum volume and MS salt strength in AdRId and AdRD suspension culture.

		AdRId		AdRD	
		Specific growth rate (x $10^{-2}\mu$)	ZER conc. (μg/g) DW	Specific growth rate (x $10^{-2}\mu$)	ZER conc. (μg/g) DW
Inoculum volume	0.5	10.8^{a}	617^{a}	8.1^{a}	$800^{a,b}$
	1.0	9.5^{b}	550^{a}	8.4^{a}	867^{a}
	2.0	7.9^{c}	720^{a}	5.8^{b}	983^{a}
	3.0	6.8^{d}	600^{a}	4.7^{c}	$700^{a,b}$
	5.0	4.0^{e}	517^{a}	2.8^{d}	717^{b}
MS salt strength	0.5	7.2^{c}	1500^{a}	5.5^{c}	2520^{a}
	1.0	11.0^{a}	600^{b}	8.4^{a}	850^{b}
	2.0	9.6^{b}	1100^{a}	7.1^{b}	1234^{b}

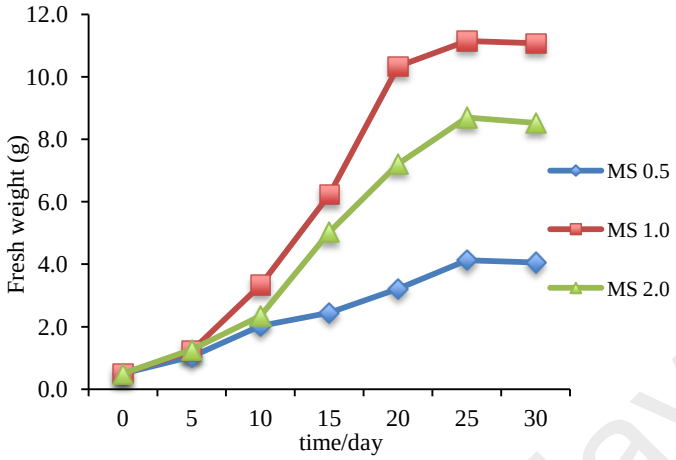

Figure 4.10a: Growth rate of AdRId in different salt strength.

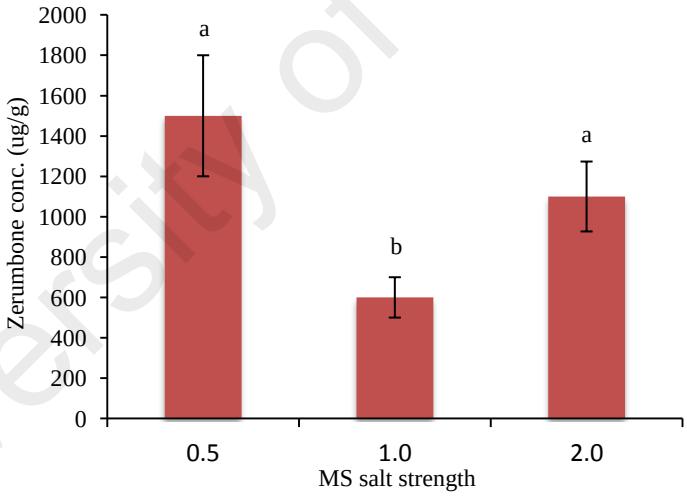

Figure 4.10b: ZER production of AdRId in different salt strength.

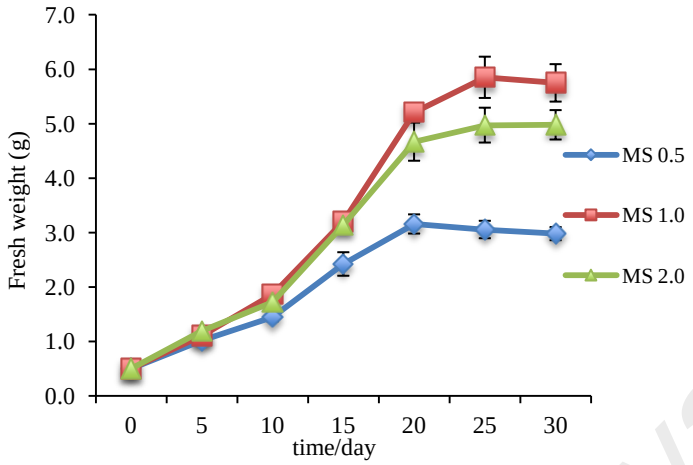

Figure 4.11a: Growth rate of AdRD in different salt strength.

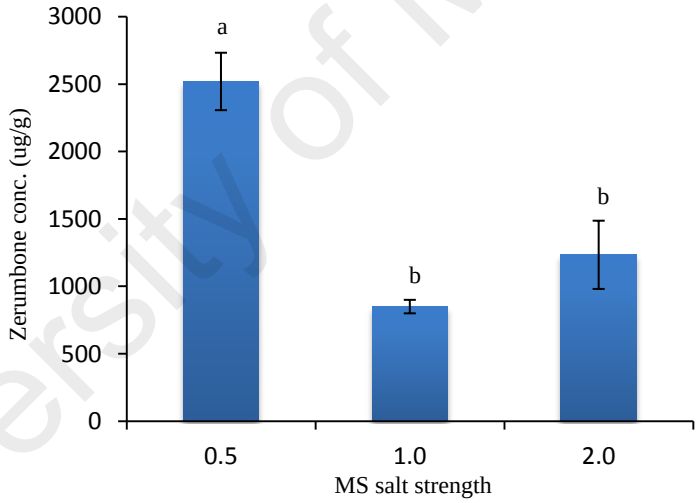

Figure 4.11b: ZER production of AdRD in different salt strength.

4.3.3 Comparison of ZER production in rhizome and adventitious root cultures

For initiation of AdR from *Z. zerumbet*, the selection of inoculum type is important especially for compound extraction. AdR initiated through direct pathway contained higher ZER compound compared to the one initiated through callus phase. This might be due to the difference in morphological and anatomical features between both roots, which contributed to the accumulation of the bioactive compound. Baque et al. (2010a)

stated that induction of callus-free, healthy adventitious roots is the key for secondary bioactive compound harvesting. Types of auxins and concentrations were found to directly affect biomass and compound accumulation. From the study, it showed that root induction was suppressed markedly with higher concentrations of NAA and IBA. While for AdR suspension culture, high biomass accumulation was achieved in moderate concentration of auxin in which NAA dominates for both AdRD and AdRId. High biomass accumulation also did not contribute to high ZER concentration per DW. High ZER concentration was achieved by increasing auxin concentration but in contrast produce low biomass accumulation. This was in accordance with findings by Fazal et al. (2014) stating that the flavonoids content in AdR culture of *Prunella vulgaris* L. did not show linear correlation with biomass accumulation. It was also noted from this study that the ZER compound was intact in the root cells as compared to cells suspension culture, whereby ZER was secreted into spent liquid media. Even though the ZER extracted from AdR was 16-fold lower than the one extracted from rhizome, other strategies could be adopted to enhance the ZER compound such as elicitation, precursor feeding and genetic engineering. For multiplication and maintenance of AdR suspension culture, growth rate of AdRId was superior to AdRD but in contrast, the accumulation of ZER was significantly higher in the latter. This could be due to high cellular activity in AdRD owing to high stain intensity which may account for higher ZER production compared to AdRId. Lowering MS salt to half the standard concentration provided two-fold ZER accumulation compared to full MS strength. This might be caused by cell stress due to low nutrient supply, which led to high accumulation of ZER. In summary, adventitious root culture is a simple technique that could be established for biomass and compound production. In AdR of *Z. zerumbet*, ZER was found intact in the root cells and this could be exploited as a model system for compound enhancement through elicitation and large scale production through bioreactor.

CHAPTER 5: ENHANCEMENT OF ZERUMBONE IN ADVENTITIOUS ROOT CULTURE OF *ZINGIBER ZERUMBET* BY METHYL JASMONATE, SALICYLIC ACID AND ITS RELATION WITH ANTIOXIDANT ENZYME ACTIVITIES

5.1 INTRODUCTION

Plants produce a wide variety of compounds that are not strictly necessary for growth and development, but have crucial roles in defence and adaptation to the environment. These secondary metabolites, which includes alkaloids, phenolics, steroids and terpenes display a broad range of biological activities and have vast potential applications in industries such as pharmaceutical, agrochemical, flavors, fragrances, color, biopesticide, and food-related (Gupta et al., 2010; Cusido et al., 2013; Ramirez-Estrada et al., 2016). The production of these valuable compounds in nature is restricted due to environmental challenges, diseases, extinction of plants attributed to over-harvesting and the requirement of a large planting area. Therefore, researchers are exploring novel routes in producing plant materials for bioactive compound harvesting, and one such approach is through biotechnological process.

The biotechnological production of secondary metabolites in plant cell and organ cultures is an attractive alternative to whole plant extraction (Skrzypczak-Pietraszek et al., 2014). Verpoorte et al. (2002) highlighted the promising technologies of producing valuable plant-specific metabolites through plant cell and organ culture systems especially adventitious roots as an alternative for conventional planting method. Nevertheless, the primary challenge in producing secondary metabolites in cell culture is the low occurrence of the compound(s) in the cells compared to developed plants. Therefore, product yields from the cell culture need to be increase in order to make it commercially viable than that of field-grown plants (Alfermann & Petersen, 1995).

One of the most reliable methods to enhance bioactive compound production from plant cell cultures is through elicitation.

5.1.1 Elicitation

Elicitation is one of the most efficient techniques currently used for improving the secondary metabolites production through biotechnological approach. Elicitors are compounds that stimulate any type of plant defense thus promoting secondary metabolism for cell and whole plants protection (Baenas et al., 2014; Ramirez-Estrada et al., 2016). Generally, the secondary metabolites accumulation in plants is due to a common response to biotic and abiotic stresses therefore their expression and accumulation, could be stimulated by biotic and abiotic elicitors (Sivanandhan et al., 2012). Elicitors have been widely employed to increase the production of secondary metabolites in plant cell cultures and considered as one of the most effective strategies to enhance the biosynthesis of these metabolites in plant cell cultures (Zhang et al., 2000; Lu et al., 2001; Wang et al., 2007).

5.1.2 General mechanism of action of elicitors

The response of plants to elicitor-induced stress generally begins at the cell plasma membrane. Besides plasmalemma membrane-associated elicitors, there are several other receptors in the cytosol associated with both nucleus and cytosolic membranes. The most studied receptors in cell culture are for plasma membrane-associated elicitors, and efforts for elicitor signal molecules isolation and corresponding receptors identification have been carried out (Ramirez-Estrada et al., 2016). In general, plant resistances to diseases are controlled by both plant resistance (*R*) and pathogen avirulence (*Avr*) genes (Mc Dowell & Woffenden, 2003) where the specific *Avr* products trigger defense responses in cultivars with the matching *R* genes. Meanwhile the action of general

elicitors can activate defenses in cultivar of more than one species (Garcia-Brugger et al., 2006). Since elicitors can induce reactions in different type of species, it appears that plants have common receptors among them. In cell plasma membranes, a number of elicitor-binding sites have been identified for a range of chemically different structured elicitors in which *R* and *Avr* gene products play a key role in this step (Garcia-Brugger et al., 2006).

The elicitor signal transduction perceived by the receptors entails the action of second messenger, which further amplify the signal for other downstream reactions. The sequentially occurring events in elicitor-induced defense responses started with elicitor perception by the receptor, followed by series of events ending with late defense response gene expression leading to secondary metabolites production and accumulation (Figure 5.1). Thus, elicitor signal transduction is a multiple component network that establishes an efficient defense by various sequential reactions and these multiple components consist of parallel or cross-linking signaling pathways leading to different target responses.

An elicitor-signaling pathway may vary in the perception of different elicitor signals or target defense responses. Recent studies revealed that defensive cellular process is generally regulated by two or more signaling pathways working in collaboration (Ramirez-Estrada et al., 2016). It is also highlighted that cross-talk among multiple signaling pathways is an imperative mechanism in plant transduction network; enabling plants to regulate different sets of genes temporally and spatially in a range of situations against many types of stress (Zhao et al., 2005).

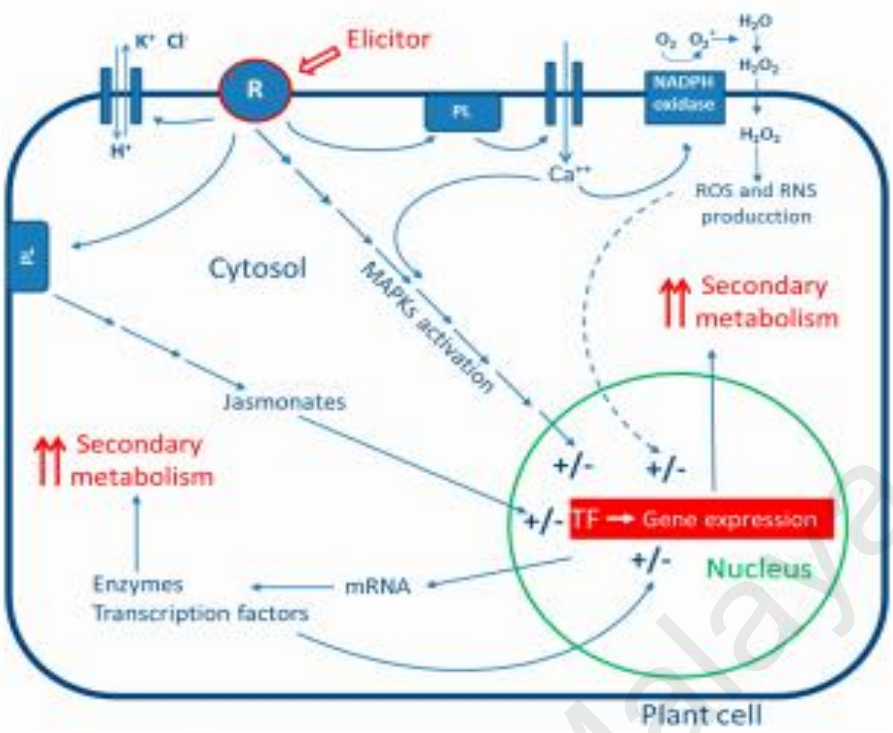

Figure 5.1: Schematic representation of the possible responses of cell to elicitation. R: receptor; PL: phospholipase; MAPKs: mitogen activated protein kinase; ROS: reactive oxygen species; RNS: reactive nitrogen species; TF: transcription factor (source: Ramirez-Estrada et al., 2016).

5.1.3 Elicitors

An elicitor could be described as a substance which, when introduced in small concentration to a living cell system, initiates or improves the biosynthesis of specific compounds. The classification of elicitors could be divided into biotic, abiotic, and plant hormones. The application of biotic, abiotic or plant hormones stimuli to plant cell cultures serve as an effective strategy to improve *in vitro* secondary metabolites production (Zhao et al., 2005; Vasconsuelo & Boland, 2007).

Abiotic elicitors can be regarded as substances of non-biological origin, mainly of inorganic compounds such as salts or other physical factors (Namdeo, 2007). Inorganic chemicals for instance salts or metal ions have been employed to increase bioactive

compounds production by modification of their plant secondary metabolism. The abiotic elicitors enhanced plant secondary metabolites in a wide variety plant species e.g. cell suspension, hairy and adventitious root culture system (Ramirez-Estrada et al., 2016).

5.1.4 Biotic elicitors

The term biotic elicitors refer to those of biological origin, which consist of endogenous and exogenous compounds. The exogenous biotic elicitors include compounds release by microorganisms and other pathogens, or formed by the action of plant enzymes on microbial cell walls, fungal and bacterial lysates, yeast extracts, and polysaccharides from microorganism cell walls such as chitin and glucans. While those of endogenous origin comprise of polysaccharides arising from pathogen degradation of plant cell wall, intracellular proteins, and small molecules synthesized by the plant cell in response to different types of stress or pathogen attack, including plant hormones such as methyl jasmonate and salicylic acid.

The application of biotic elicitors in plant cell cultures comprises an excellent system to enhance secondary compounds production with phytoalexinic properties, as well as to acquire more insight into the regulation of their biosynthetic pathways. The majority of biotic elicitors are recognized by specific receptors bound to the cell membrane. Signal transduction system will then transfer the stimulus is to the cell, producing changes that finally lead to the formation of phytoalexin, and the response of the plant is determined by several factors, mainly the genetic characteristics and physiological state of the plant (Baenas et al., 2014).

Jasmonic acid and salicylic acid are recognized as the main signaling molecules involved in the plant defense response and therefore, are widely used as chemical inducers for *in vitro* secondary metabolite production (Goyal & Ramawat, 2008). Bulgakov et al. (2002) also stated that among the plant-derived elicitors that mediate the signal transduction involved in plant defense responses is methyl jasmonate (MeJA) and salicylic acid (SA). Exposure to MeJA and SA often leads to the generation of reactive oxygen species (ROS), which sequentially react with cellular components, triggering an immediate plant defense response (Mueller et al., 1993; Lamb & Dixon, 1997; Zhao et al., 2001b; Suhita et al., 2004). Meanwhile, both MeJA and SA are able to stimulate secondary bioactive compound production in many plants species (Taguchi et al., 2001; Kang et al., 2004; Zhou and Wu, 2006; Vasconsuelo & Boland, 2007).

5.1.4.1 Methyl jasmonate

The widely used chemical elicitor, jasmonates, which include jasmonic acid (JA) and methyl jasmonate (MeJA), are a family of cyclopentanone compounds that modulate a broad range of plant responses (Creelman & Mullet, 1997) and act as effective elicitors in enhancing *in vitro* secondary metabolites production. Jasmonic acid (JA) is an important signaling molecule of plant in response to wound and pathogen attack. Jasmonic acid and its volatile methyl ester namely methyl jasmonate are a few best-known candidates for elicitor-induced plant secondary metabolite biosynthesis (Gutierrez et al., 2012). Zhao et al. (2005) suggested the possibility of jasmonic acid as a link between the elicitor-receptor complex and gene activation, which in turn is accountable for the induction of important enzymes involved in the phytoalexin synthesis. The effect of adding jasmonates has been investigated in hairy root propagation of *Catharanthus roseus* with a several-fold increased in alkaloid content (Rijhwani et al., 1998). Besides, jasmonic acid was also observed to accumulate within

the tissue in response to several other elicitors (Gundlach et al., 1992). Although MeJA is an ester of jasmonic acid, its effects might vary and it has been suggested that MeJA diffuses into the cytoplasm, where it is hydrolyzed by esterases to jasmonic acid, which was previously stated acts as a secondary messenger in the defense mechanism against pathogens (Ryan & Farmer, 1990; Gundlach et al., 1992).

MeJA had become a powerful candidate for its role in systemic signaling and this compound could diffuse to distal parts of the plant *via* the vapor phase or by intercellular migration, possibly through the phloem. The plant floral scent MeJA has been identified as a crucial cellular regulator that mediates diverse developmental processes and defense responses against biotic and abiotic stresses (Cheong & Choi, 2003). Plants that being attack by microbial pathogens, or are mechanically damaged, produce high levels of signaling molecules such as jasmonic acid (JA) and methyl jasmonate (MeJA), which accumulate in the damaged parts of the plant and are instrumental in metabolism re-programming (Shulaev et al., 2008). The jasmonate signaling pathway is connected to other signaling pathways, thus forming a complex regulatory network. The genes being up-regulated by MeJA treatment include those involved in jasmonate biosynthesis, secondary metabolism and those encoding stress-protective and defense proteins (Cheong & Choi, 2003).

JA and its more active derivative MeJA can stimulate the production of a broad range of plant secondary metabolites such as rosmarinic acid, terpenoid indole alkaloid and plumbagin in various cell cultures (Krzyzanowska et al., 2012; Silja et al., 2015). JA and MeJA have been used as elicitors for stilbene biosynthesis in *Vitis vinifera* foliar cuttings (Belhadj et al., 2006), *Vinis vinifera* cell cultures (Tassoni et al., 2005) and *V. rotundifolia* hairy root cultures (Nopo-Olazabal et al., 2014). The addition of MeJA to

V. vinifera cell cultures also promoted anthocyanin accumulation (Tisserant et al., 2016). Studies suggested that elicitor action by exogenous MeJA on the medicinal plant, *Centella asiatica* (L.) leads to dynamic metabolic changes that involve alterations in some steps of the terpenoid pathway, with an increase in triterpenoids levels (Mangas et al., 2006; Kim et al., 2010; James et al., 2013 In addition, Zhang et al. (2007) reported that MeJA treatment amplified tropane alkaloids production in transgenic *Hyoscyamus niger* hairy root cultures.

Elicitation studies have also shown that MeJA can escalate the production of peruvoside, a cardiac glycoside in cell suspension culture of *Thervetia peruviana* (Zabala et al., 2009) and phenolic compounds accumulation in hairy root cultures of *Scutellaria lateriflora* (Marsh et al., 2014). Likewise, MeJA treatment of *Catharanthus roseus* hairy roots culture stimulated the synthesis of catharanthine, one of the two precursors of the anticancer compound vinblastine. Studies with MeJA-elicited *Centella asiatica* hairy roots and cell suspension culture also resulted in highly increased centellosides production (Kim et al., 2007; Bonfill et al., 2010). The content of valerenic acid in hairy root culture of *Valeriana officinalis* was also tremendously increased through treatment with methyl jasmonate (Torkamani et al., 2014). Synergistic effect was observed when MeJA was combined with SA and acetylsalicylic acid, which reported to stimulate the tropane alkaloid yields in the transgenic *Atropa baetica*, which overexpressed the *hyoscyamine 6*β*-hydroxylase* (*h6h*) gene (Jaber-Vazdekis et al., 2008).

5.1.4.2 Salicylic acid

Salicylic acid (SA) is a monohydroxybenzoic acid, a type of phenolic phytohormone and is widely used in organic synthesis. It is derived from the metabolism of salicin and functions in plant growth and development, photosynthesis, transpiration, ion uptake and transport. SA is a small molecule with an imperative role in plant defense regulatory systems, known to induce systemic acquired resistance (SAR) to many pathogens (Pieterse & van Loon, 1999; Hayat et al., 2010). SA is also involved in endogenous signaling, mediating in plant defense against pathogens (Hayat & Ahmad, 2007). The signal of plant defense could also being transferred to nearby plants by conversion of salicylic acid into volatile ester namely methyl salicylate. During the plant-pathogen interaction, a rapid SA accumulation in the infected site activates a hypersensitive response in which the signals will subsequently spreads to other plant parts inducing a wide range of defense responses including plant secondary metabolites production (Wasternack & Hause, 2013). This is the reason of SA being widely applied as secondary metabolism elicitor (Namdeo, 2007; Dučaivá et al., 2013).

Treatment with exogenous SA was previously reported to induce secondary metabolites production in many plant species, for instant anthraquinones in *Rubia cordifolia* (Bulgakov et al., 2002), soluble phenolic compounds in *Matricaria chamomilla* and *Salvia miltiorrhiza* (Kováčik*et* et al., 2009; Dong et al., 2010), podophyllotoxin in *Linum album* (Yousefzadi et al., 2010), artemisinin in *Artemisia annua L.* (Pu et al., 2009) and aloe emodin and chrysophanol in *Aleo vera* (Lee et al., 2013). In SA-treated *Linum album* cell cultures, the production of podophyllotoxin was three-fold higher than in control cultures after three days of supplementation (Yousefzadi et al., 2010). Likewise, the resveratrol accumulation in the leaves of *Arachis hypogaea* increased after SA treatment, and the STS expression levels were

also up regulated (Chung et al. 2003). In *Ginkgo biloba* cell suspension cultures, SA addition as well induces sesquiterpenes accumulation, including bilobalide and ginkgolide *A* and *B* (Kang et al., 2006). While work by Sivanandhan et al. (2012) on adventitious roots of *Withania somnifera* also displayed an increase in anti-inflamatory withanolides production after SA treatment. The production of taxol, an anticancer compound was also successfully enhanced by SA in *Corylus avellana* cell cultures (Razaei et al., 2011). Various studies on SA elicitation to enhance diterpene alkaloid production were also done on the Taxus genera. Wang et al. (2007) treated *T. chinensis* cell suspension cultures with an optimal concentration of 20 mg/L SA to induce paclitaxel production. Improved yields of taxane were also achieved in a two-stage cell suspension culture of *T. baccata* using two different concentrations of SA (Khosroushahi et al., 2006). Recent study by Li et al. (2016) also showed approximately three-fold increase of total flavonoid content in *Glycyrrhiza uralensis* Fisch. when treated with salicylic acid.

Synergistic effects of SA with other treatment were also capitalized in optimizing plant secondary compound production. Investigation of combining SA with magnetic field showed an improve in paclitaxel yield of *T. chinensis* cell suspension cultures (Fan et al., 2006) and recently, Xu et al. (2015) discovered the production of stilbene in *Vitis vinifera* cell suspension culture was positively affected by SA, alone or in combination with other treatments.

5.1.5 Oxidative stress

A change in environmental condition of plants such as excess light, cold or heat, drought, invasion by pathogenic microorganisms and oxidizing atmospheric pollutant lead to an increase in reactive oxygen species (ROS). ROS are mandatory by-products of various metabolic pathways related to an aerobic lifestyle (Davies et al., 1995; Hampton et al., 1998; Lamberth, 2004). Reactive oxygen species (ROS) are formed in biological system as part of normal metabolism and an increase in ROS resulted in oxidative stress (OS) condition.

The ROS consist of both free radical oxygen intermediates such as peroxide anions (O^{-2}) and hydroxyl radicals (OH^{-}), and non-free ones including hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl) (Halliwell, 2001). The ROS can react instantaneously and non-specifically with essential biological molecules and lead to cellular functions alteration (Montibulus et al., 2013). The excessive augmentation of ROS would impose severe morphological, metabolic, and physiological anomalies in plants ranging from chlorosis of shoots to lipid peroxidation, DNA mutation and protein degradation (Emamverdian et al., 2015).

To mitigate the harmful effects of free radicals, plant cells have developed antioxidant defense mechanism composed of enzymatic antioxidants like superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), guaiacol peroxidase (GPX) and glutathione reductase (GR) and non-enzymatic antioxidants like ascorbate (AsA), glutathione (GSH), carotenoids, alkaloids, topopherols, proline and phenolic compounds (flavonoids, tannins and lignin) that act as the scavengers of free radicals (Foyer et al., 1994; Michalak, 2006; Rastgoo et al., 2011; Sharma et al., 2012). Besides

their implication in intracellular signaling, ROS also play a central role in molecular cross-talked which related to oxidative stress and secondary metabolism.

5.1.5.1 Hydrogen peroxide

Hydrogen peroxide is the most stable ROS compared to superoxide, hydroxyl and singlet oxygen. It is a week acid which lacking of unpaired electrons (non-radical). The lifetime of H_2O_2 in tissue is short due to the activities of catalase and peroxidases that putrefy this substance (Halliwell & Gutteridge, 2007). Detection of H_2O_2 could be made from few seconds to several days after application of stress factor and this difference could be caused by many factors. H_2O_2 is probably produced in specific area (hot spot) for example the tip of growing root hairs, which means that total tissue/organisms H_2O_2 activity is very low (Foreman et al., 2003).

5.1.6 Defense against oxidative stress

Peroxidation of membrane lipids is caused by ROS, which react with unsaturated fatty acids, leading to cellular contents leakage and hence damaging the cells (Smirnoff, 2000). To avoid such damage caused by the excess ROS, plants have developed a biological defense mechanism to scavenge ROS. Plants have a number of strategies to withstand the oxidative stress (Gill & Tuteja, 2010).

The first strategy is activation and *de novo* synthesis of antioxidant (defense mechanisms to scavenge ROS) involving the antioxidant enzymes e.g. catalase (CAT), ascorbate peroxidase (APX), superoxide dismutase (SOD) and non-enzymatic small antioxidant molecules such as glutathione, ascorbic acid and carotenes (Harris, 1992). Secondly, plants probably synthesize protein isoforms and lipids which are less sensitive to oxidation; thirdly, most plants probably protect living tissues from stresses

by layer of dead cells which died rapidly by programmed ROS-induced mechanisms and finally, plants activate biosynthesis systems for compensation of damaged components. Zhang et al. (2014) stated that under NaCl stress, the antioxidant system of *Limonium sinense* is activated and effectively scavenges ROS which reduces oxidative damage and allows the plant to maintain their growth. Catalase showed the largest increase in activity while peroxidase and superoxide dismutase also increased in their activities.

5.1.6.1 Enzymatic system

Antioxidant enzyme plays crucial role in the scavenging of reactive oxygen species (ROS) during plant metabolism (Camp et al., 1997; Das & Roychoudhury, 2014). Antioxidant enzyme activities are inducible by oxidative stress (Foyer et al., 1994). Ferreira et al. (2010) stated that an antioxidant compound could delay or inhibit oxidation of lipids and other molecules by inhibiting the initiation or propagation of oxidizing chain reaction. Catalases play a vital role in defense against oxidative stress by catalyzing the breakdown of H_2O_2 into oxygen and water whereas SOD reduces O^{2-} to H_2O_2, which further reduced to H_2O by catalases in peroxisomes and by APX in the chloroplast and cytosol. In plant cells, APX specifically uses ascorbate as a physiological reductant and is considered a crucial component in the metabolic defense against oxidative stress (Asada, 1992; De Gara et al., 2000). APX has high substrate specificity for ascorbate and is the primary H_2O_2 scavenging enzyme in the chloroplasts and cytosol of the plant cell (Asada, 1992; Gest et al., 2013). It operates by a series of coupled redox reactions, both in photosynthetic and non-photosynthetic tissues (Asada, 1994; Arrigoni, 1994).

There are four isoforms of APX that have been isolated, one located in the cytosol, two in the chloroplast (Mittler & Zilinskas, 1991) and the fourth isoform of APX was identified in glyoxysomal membranes of pumpkin (*Cucurbita pepo*) and cotton (*Gossypium hirsutum*) (Yamaguchi et al., 1995; Bunkelmann & Trelease, 1996). As for chloroplast-contained APX, one soluble form of APX is in the stroma (Chen & Asada, 1989) and the other one is thylakoid-bound form (Miyake & Asada, 1992).

5.2 MATERIALS AND METHODS

5.2.1 Plant material and culture conditions

The initiation and maintenance of adventitious root culture were performed in an optimized medium and according to culture conditions described in section 4.2.4 and 4.2.5, Chapter 4. Adventitious root culture of *Z. zerumbet* was developed through direct organogenesis from the slices of shoot bud meristem and cultured on optimized media consisting of Murashige and Skoog (MS) basal medium supplemented with 2 mg/L NAA, 3% sucrose and 2 g/L phytagel. After four weeks of culture, well-established adventitious roots were separated and transferred to liquid media for the induction of root suspension culture. The adventitious root (AdR) suspension culture was maintained in an optimized liquid media consisting on Murashige and Skoog (MS) basal medium supplemented with 1 mg/L NAA and 3% sucrose. The AdR suspension cultures were routinely sub-cultured every two weeks and incubated in the 16 hours/day light regime under continuous shaking at 80 rpm. All media were adjusted to pH 5.7 prior to autoclaving and culture temperature was maintained at 25 ± 1°C.

5.2.2 Elicitation of adventitious roots

Elicitation of AdR was done by using different concentrations of MeJA and SA at 100, 200, 400, 800, 1200 μM. AdR without elicitation was used as control. The AdR was incubated for 21 days for biomass accumulation due to nearly complete root growth at the end of exponential phase and therefore bioactive compound production could take place at the best possible (Sivanandhan et al., 2012). The zerumbone titre was determined every 5 days for a period of 30 days. A stock solution of MeJA and SA was prepared separately by each substance dissolution in distilled water and then filter-sterilized by using 0.22 μm filters (Sartorius) before its addition into root suspension cultures. Meanwhile, for untreated sample, equal volume of sterile distilled water was added to the cultures as blank treatment control for the different elicitor concentrations studied. All experiments were carried out in triplicate cultures and each treatment was repeated three times.

5.2.3 Effect of MeJA supplementation time on cell growth and zerumbone production

Following the above investigation, MeJA was selected for further study. The appropriate MeJA supplementation time into AdR culture is known to be crucial in order to increase production. MeJA (800 μM) was added to the cell culture at 15, 20 and 25 days of culture. Subsequently, the zerumbone (ZER) concentration was determined for every two days for a period of 20 days.

5.2.4 Preparation of extract for analysis of zerumbone

The preparation of extract for ZER analysis was done based on procedure described in section 4.2.6.2, Chapter 4.

5.2.5 Identification of zerumbone compound

The identification of ZER compound was done according to procedure in described in section 3.2.9, Chapter 3.

5.2.6 Protein content analysis

Adventitious root weighing 0.02 gram was ground in liquid nitrogen using chilled pestle and mortar, and then homogenized in 1.0 ml 50 mM potassium phosphate buffer (pH 7.0) containing 2% (w/v) insoluble polyvinylpolypyrrolidone (PVP), 1.0 mM ethylenediamine tetraacetic acid (EDTA) and protease inhibitor. After centrifugation at 10 000 rpm for 10 minutes, the supernatant was collected and used for enzyme assays. The soluble protein content was measured at 595 nm according to the method of Bradford (1976) using bovine serum albumin (BSA) as standard. All experiments were carried out in triplicate.

5.2.7 Biochemical assays

5.2.7.1 Hydrogen Peroxide

Hydrogen peroxide concentration was determined according to Velikova et al. (2000) with slight modification. 0.02 gram of AdR culture was ground in liquid nitrogen using chilled pestle and mortar and then homogenized in 1.0 ml 0.1% (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 10 000 rpm for 10 min and 0.25 ml of the supernatant was added to 0.25 ml of 50 mM potassium phosphate buffer (pH 7.0) and 0.5 ml of 1.0 M potassium iodate (KI). Absorbance of the supernatant was read at 390 nm using UV-Vis spectrophotometer V-630 (Jasco, Japan). The concentration of H_2O_2 was read-off from a standard calibration.

5.2.7.2 Ascorbate Peroxidase (APX)

The ascorbate peroxidase (EC 1.11.1.11) activity was measured based on the method of Nakano and Asada (1981). The reaction mixture consisted of 0.1 ml of crude enzyme extract, 0.6 ml of 50 mM sodium phosphate buffer pH 7, 0.1 ml of 5.0 mM ascorbate, 0.1 ml of 1.0 mM hydrogen peroxide and 0.1 ml of 1.0 mM EDTA in a final volume of 1.0 ml. The reaction was started after hydrogen peroxide addition, and the activity was followed by the decrease in absorbance of H_2O_2 at 290 nm for 10 min. The molar extinction coefficient 2.8 $mmol^{-1}$ cm^{-1} was used to calculate ascorbate peroxidase activity according to equation (1) as shown below:

Equation 1

$$\text{Ascorbate peroxidase activity}\,(\text{U mL}^{-1}) = \frac{\Delta Abs_{290\text{nm}} \text{ x } V_s}{\xi_{290\text{nm}} \text{ x } l \text{ x } V_{ls}}$$

where

$\Delta Abs_{290\ nm}$ = Absorbance difference at 290 nm at for a specific interval (min^{-1})

$\xi_{290\ nm}$= Extinction coefficient of ascorbate i.e. 2800 M^{-1} cm^{-1}

V_s = Volume of substrate (mL)

V_{ls} = Total assay volume (mL)

l = path length of cuvette (1 cm)

5.2.7.3 Catalase (CAT)

The catalase (EC 1.11.1.6) activity was measured spectrophotometrically according to Aebi (1984). The assay mixture consisted of 30 mM H_2O_2 in 50 mM sodium phosphate buffer (pH 7.0) and 0.1 ml of crude enzyme extract in a final volume of 3.0 ml. To ensure proper mixing of the solution, a tiny magnetic stirrer bar to provide gentle agitation was placed at the bottom of the cuvette. The decomposition of H_2O_2 was monitored continuously using molar extinction coefficient of 43.6 $M^{-1}cm^{-1}$ at 240 nm for 2 minutes. One unit (U) of catalase activity is defined as 1mol H_2O_2 decomposed per minute at 25°C. The catalase activity was calculated according to equation (2) as shown below:

Equation 2

$$\text{Catalase activity}\,(\text{U mL}^{-1}) = \frac{\Delta Abs_{240\text{nm}} \text{ x } V_s}{\xi_{240\text{nm}} \text{ x } l \text{ x } V_{ls}}$$

where,

$\Delta Abs_{240\text{ nm}}$ = Absorbance difference at 240 nm at for a specific interval (min^{-1})

$\xi_{240\text{ nm}}$= Extinction coefficient of catalase i.e. 43.6 M^{-1} cm^{-1}

V_s = Volume of substrate (mL)

V_{ls} = Total assay volume (mL)

l = path length of cuvette (1 cm)

5.2.8 Statistical Analysis

Experimental data were analyzed using one way ANOVA followed by Tukey's test at probability level of $P< 0.05$.

5.3 RESULTS AND DISCUSSION

5.3.1 The effect of elicitor concentration on biomass and zerumbone production

In the present study, the signaling compounds methyl jasmonate (MeJA) and salicylic acid (SA) were used to enhance the production of ZER in *Z. zerumbet* adventitious root (AdR) culture. Based on the results obtained for 100 μM concentration of elicitors, it was observed that there were no significant increases in ZER for all samples treated with both elicitors at different collection time compared to untreated control (Figure 5.2A). There were negligible differences in the compound titres between untreated and treated samples at 46 day after culture (maximum titre at 900 μg/g). In terms of biomass harvested, although there was a slight decline from day 26 to 41, the biomass level remained constant thereafter (Fig. 5.2B). In general, treatment with 100μM elicitors did not showed significant decrease in biomass level compared to untreated roots, which was attributed to low dosage of elicitors insufficient to affect the root growth.

At 200 μM, SA was better than MeJA. Treatments with 200 μM MeJA did not increase zerumbone accumulation level compared to SA. With 200 μM SA, it showed significantly increased zerumbone production between day 31 to 41 (Fig. 5.3A), with the highest accumulation achieved on day 41 (1016.7 μg/g DW). Even though treatment with 200 μM SA gave the highest ZER, the biomass harvested was significantly lower in comparison to untreated control and 200 μM MeJA treatments collected on the same day (Figure 5.3B). This was in accordance with finding by Ali *et al.* (2006) who observed lower biomass accumulation in *P. ginseng* adventitious roots treated with SA at similar concentration to MeJA.

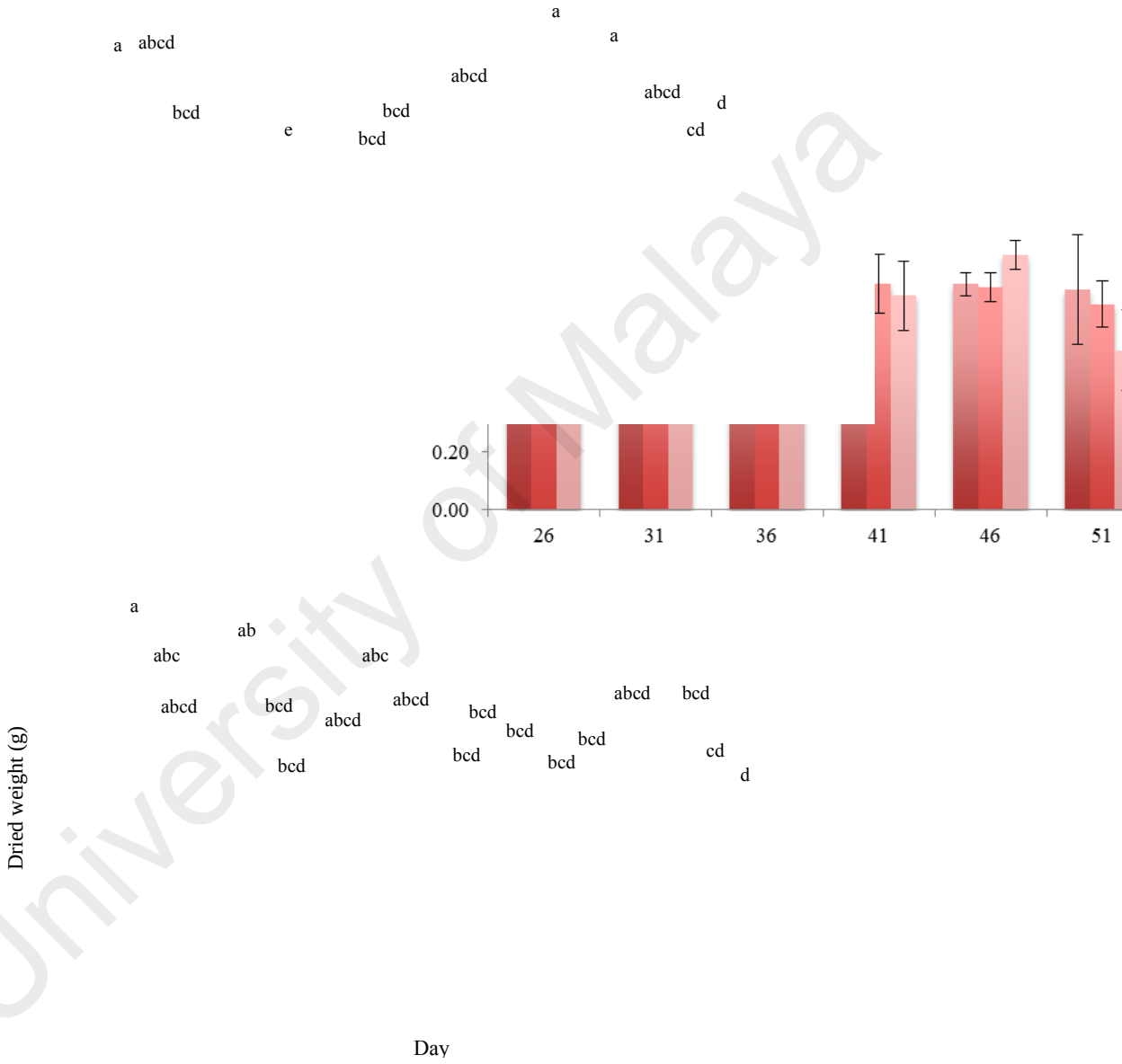

Figure 5.2: The effect of 100 µM of MeJA and SA on zerumbone production and AdR biomass of *Zingiber zerumbet* for supplementation at day 21. A : zerumbone; B : biomass. Error bars indicate standard deviations of the mean values.

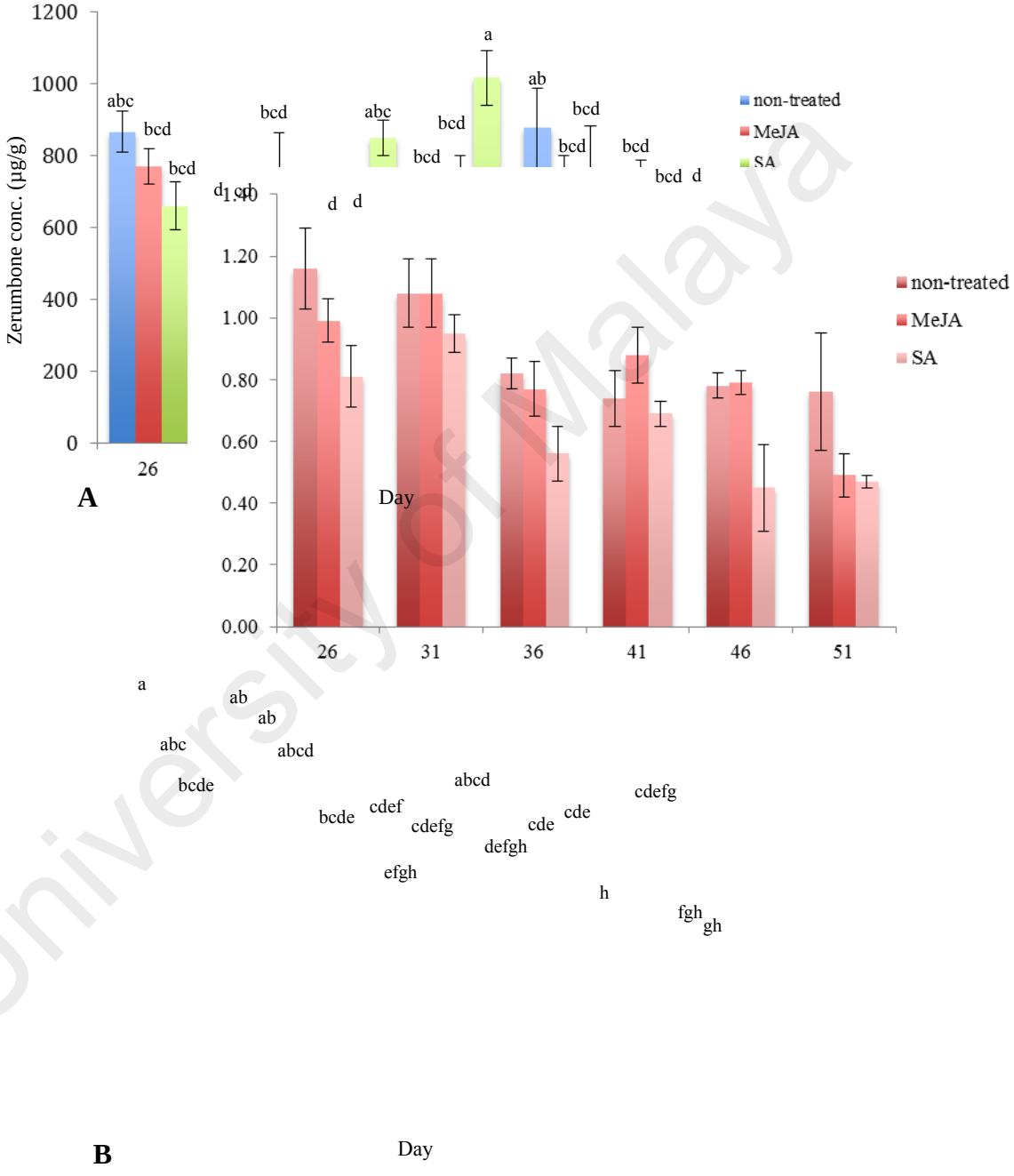

Figure 5.3: The effect of 200 µM of MeJA and SA on zerumbone production and AdR biomass of *Zingiber zerumbet* for supplementation at day 21. A : zerumbone; B : biomass. Error bars indicate standard deviations of the

In the case of 400 μM elicitor concentration, SA was superior compared to MeJA for zerumbone production. This observation agreed with finding by Lee et al. (2013) who found that, aloe emodin and chrysophanol production was increased by all of the elicitors studied, but SA remarkably elevated the level of aloe emodin and chrysophanol by more than 10-11 and 5-13 folds respectively. In other study, SA infiltration into entire *Vitis vinifera* berries also enhanced the accumulation of PAL mRNA, as well as and the synthesis of new PAL protein and enzyme activities (Chen et al. 2006).

In this study, treatment with 400 μM MeJA showed insignificant increase in zerumbone production in comparison to untreated control at day 26. The highest zerumbone production for 400 μM SA elicitation was achieved on day 41 at 3720 μg/g dry weight, representing an increase of five-fold compared to untreated control (Figure 5.4A). The HPLC profile was shown in Appendix F. A pronounced increase in zerumbone production profile was observed from day 26 to day 41 but declined significantly thereafter until day 51 (Figure 5.4A). This finding was in contrast with study by Torkamani et al. (2014) that indicated SA treatment did not significantly increase the production of valerenic acid in hairy roots of *Valeriana officinalis*. In this study, even though 400 μM SA resulted in significantly higher zerumbone accumulation, this was achieved at a longer incubation period of 41 days, which was practically time consuming and requires high production cost. In addition, the advantage was offset by significantly lower biomass level for 400 μM SA treatment (Figure 5.4B).

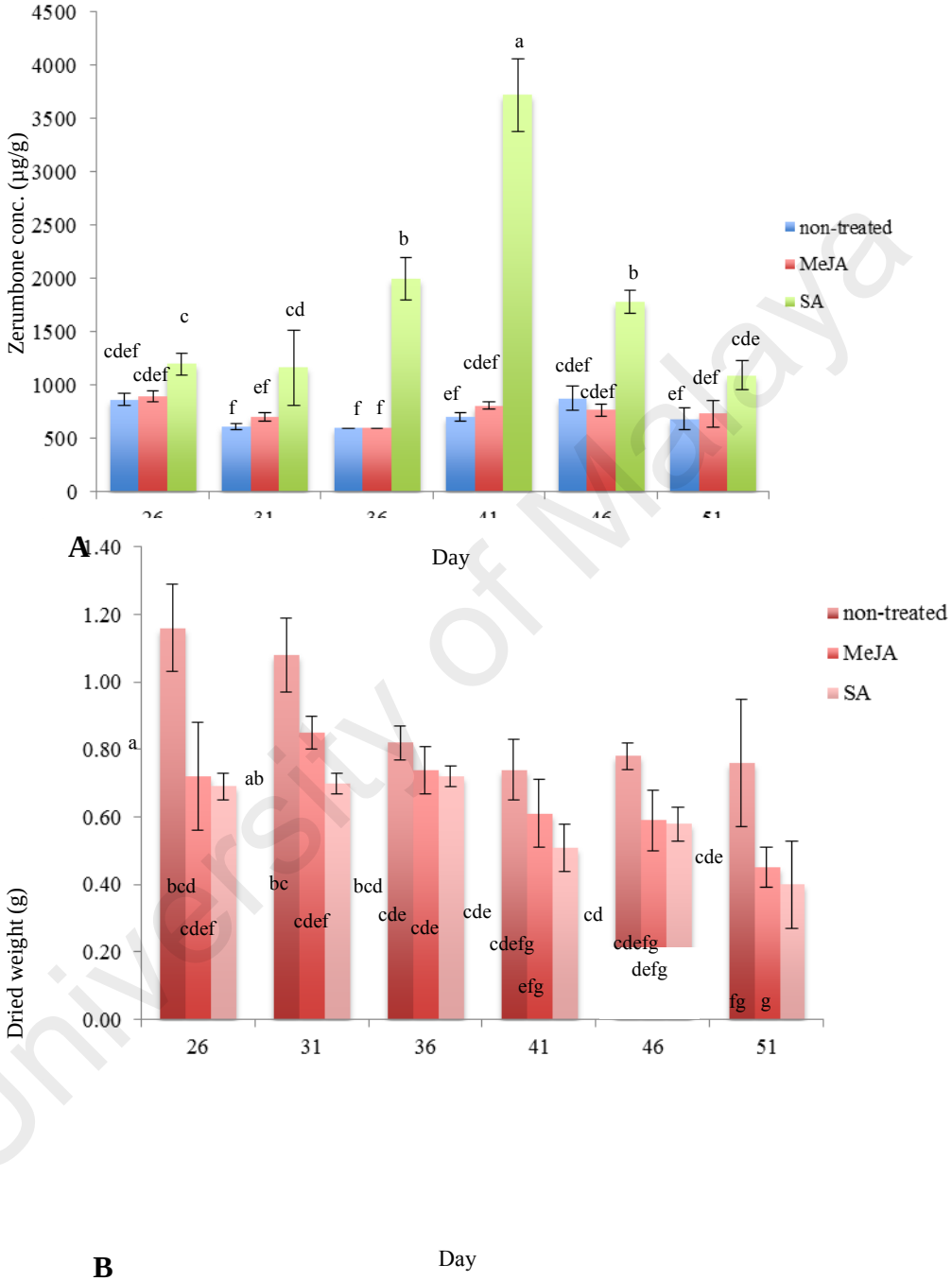

Figure 5.4: The effect of 400 µM of MeJA and SA on zerumbone production and AdR biomass of *Zingiber zerumbet* for supplementation at day 21. A : zerumbone; B : biomass. Error bars indicate standard deviations of the mean values.

In elicitation using 800 μM elicitors, MeJA showed significantly higher zerumbone production on day 26 (1300 μg/g DW) compared to untreated control and SA treatments (Figure 5.5A). The HPLC profile was shown in Appendix G. This finding was in agreement with Chodisetti *et al*. (2015) that showed MeJA yielded the maximum gymnemic acid content whereas SA evoked a moderate response. The effectiveness of MeJA in elicitation was further supported by the study done on *Salvia miltiorrhiza* G50 line which showed that total tanshinone content was increased by three-fold compared to 1.6-fold after SA treatment relative to the control (Hao et al., 2014). However, research by Lee et al. (2013) observed lower concentration of MeJA (500 μM) steadily increased and spiked endogenous levels of aloe emodin and chrysophanol with over 4-7 fold and 3-5 fold increase respectively. In blueberry *Vaccinium corymbosum* var 'Blueray', treatment with MeJA promoted an increase in anthocyanin pigments as well as the antioxidant potential in fully ripe berries. The methyl jasmonate is, in some cases, an effective elicitor of phenolic metabolism and gene expression in blueberry, though at different intensity between cultivars (Cocetta et al., 2015).

In this study, the highest zerumbone accumulation for 800μM elicitation was achieved on day 41 for elicitation with SA (1983.3 μg/g DW) with 2.8-fold increase relative to untreated control (Figure 5.5A). Even though the yield of zerumbone with SA elicitation at day 41 was significantly higher than the compound yield of MeJA elicitation at day 26, it was time consuming and the biomass level obtained was three-fold lower (Figure 5.5B). This could be due to high SA concentration exerting negative effects on biomass growth. In general, there was a gradual decline in biomass level for all treated samples and untreated control in this experiment. It was also clear that the culture duration needs to be taken into consideration when selecting the best concentration of elicitors for further study.

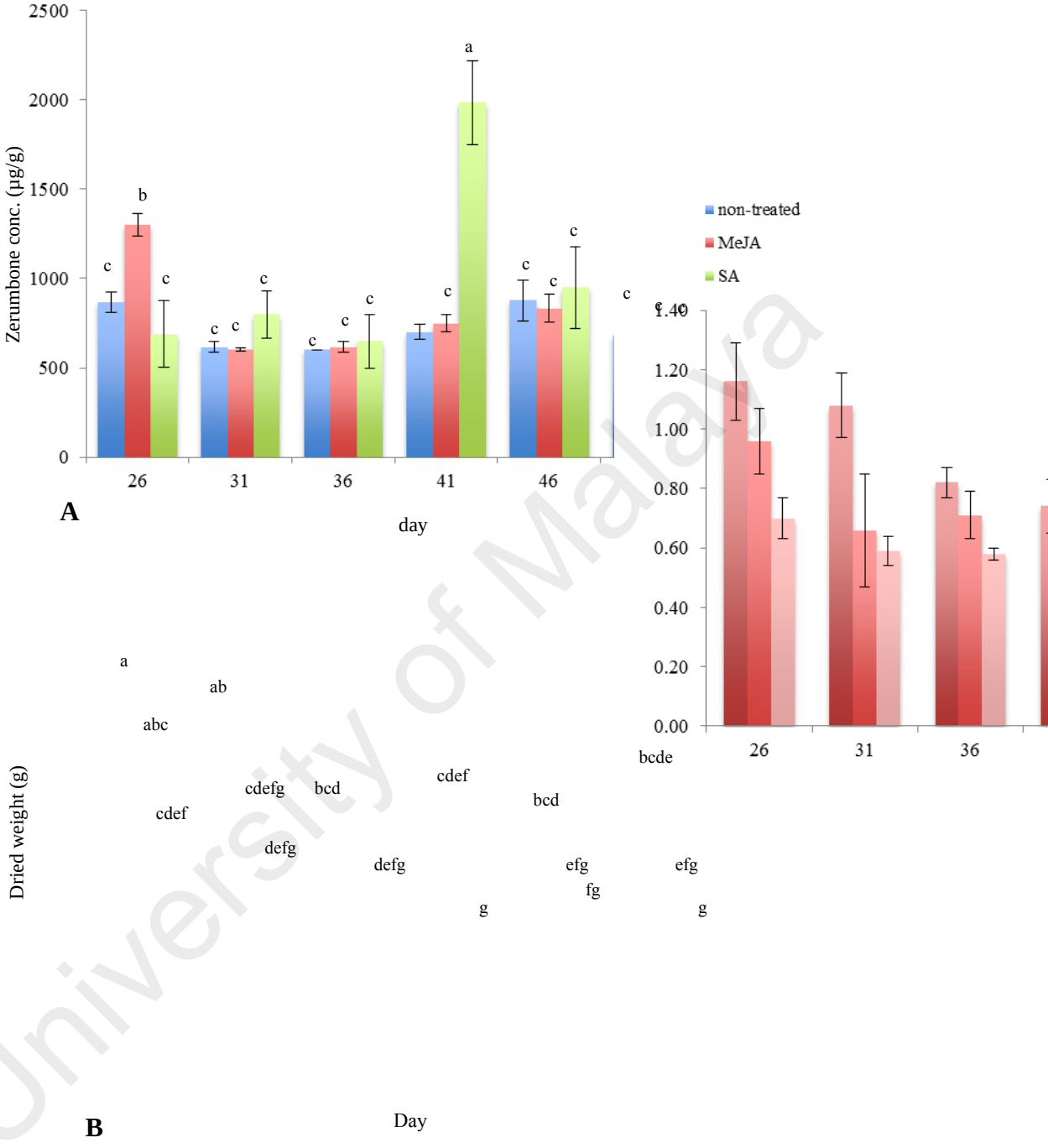

Figure 5.5: The effect of 800 µM of MeJA and SA on zerumbone production and AdR biomass of *Zingiber zerumbet* for supplementation at day 21. A : zerumbone; B : biomass. Error bars indicate standard deviations of the mean values.

Increasing the elicitor concentration to 1200 μM showed not significant increased in ZER production (Figure 5.6A). Treatment with high concentration of elicitors significantly decrease the root biomass level compared to untreated control (Figure 5.6B). Generally, supplementation of MeJA and SA at 1200 μM failed to promote further increase in zerumbone production. This could be due to inhibition effect arising from high concentration of MeJA and SA towards zerumbone production in *Z. zerumbet* adventitious root culture.

For this study, in general ZER in AdR culture showed an increment from the one without the application of elicitors. SA elicitation was more efficient at lower concentration compared to MeJA for zerumbone expression from AdR of *Z. zerumbet*. This was in agreement with finding by Jeong et al. (2005) who stated ginsenoside production was improved by SA elicitation in *Panax ginseng* hairy roots culture at a lower concentration. However, this was in contrast with finding by Lee et al. (2013), who stated that elicitation to a 35-day-old *Aloe vera* adventitious roots cultured with MeJA, SA, and ethaphon in various concentrations revealed maximum aloe emodin and chrysophanol production in adventitious roots treated with 1000-2000 μM SA.

Treatments with lower concentrations of MeJA at 100, 200 and 400 μM were insufficient to elicit high ZER production. Even though SA was superior to MeJA in most of the treatments, the biomass harvested for SA-treated roots was significantly lower to MeJA-treated roots. This could be due to their differences in signal transduction, where the MeJA concentration in plants usually increases with insect and/or animal invasions and wounding responses whereas SA is usually involved in systemic acquired resistance to microbial pathogen (Hartman et al., 2016).

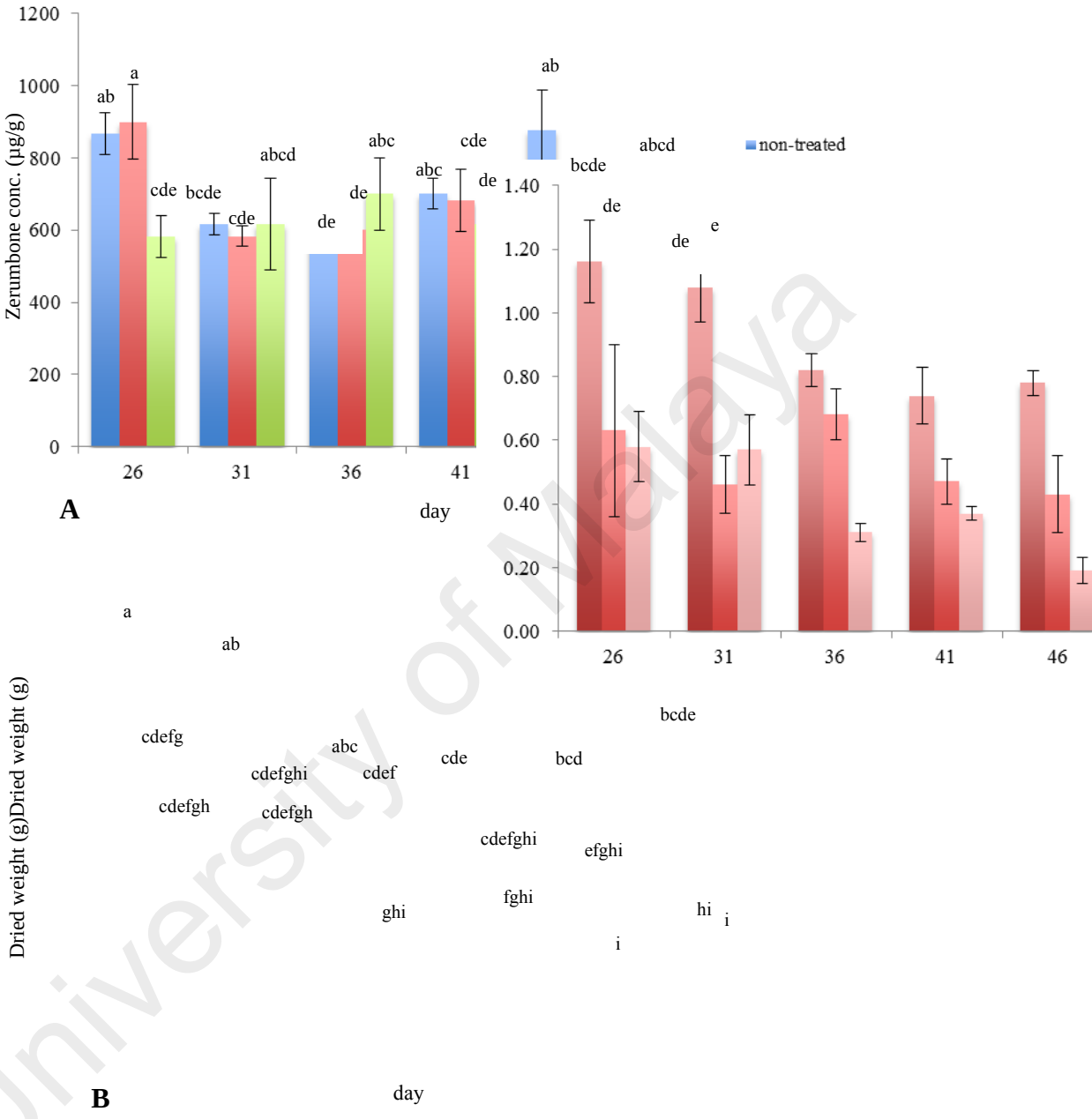

Figure 5.6: The effect of 1200 µM of MeJA and SA on zerumbone production and AdR biomass of *Zingiber zerumbet* for supplementation at day 21. A : zerumbone; B : biomass. Error bars indicate standard deviations of the mean values.

Therefore, the responses to root growth under SA elicitation were more inhibited than those under MeJA elicitation (Dong et al., 1998; Lee et al., 2015) which could be due to different activation mechanism (Schweiger et al., 2014). Other studies have proven the effectiveness of elicitors in enhancing secondary compound production. Chodisetti et al. (2015) stated that the gymnemic acid content was 15.4-fold higher than the control cultures without elicitor in *Gymnema sylvestre* cell suspension culture. This was further supported by report on the efficiency of MeJA and SA treatments in enhancing the tanshinone content in *Salvia miltiorrhiza* cell cultures (Hao et al., 2014). The efficiency of elicitation was attributed to synergistic effects of elicitors. Xu et al. (2015) recommended that the combined treatment of UV-C with MeJA or SA can be used as an efficient technique to enhance stilbene production as well as total phenolics and total flavonoids contents in *Vitis vinifera* cell suspension cultures.

5.3.2 Effects of MeJA supplementation time on biomass and zerumbone production

The elicitor induction time is one of the key factors that affect cell growth and product yield for plant cell culture. In this study, determination of supplementation time was done on selected concentration and type of elicitors based on previous experiments. Determination on the production of ZER for AdR was done with 800 μM MeJA at different supplementation times. This concentration was chosen due to moderate production of biomass and ZER which could be enhanced by elicition. The effects of MeJA supplementation time on cell growth and ZER production was evaluated by the addition of 800 μM MeJA into root cultures during exponential phase (day 15 and 20) and early stationary phase (day 25). Based on the results obtained, MeJA treatment during exponential phase (day 15 and 20) led to significant increase in ZER expression. For day 15, significantly high zerumbone production at 42777 μg/g dry weight was

observed at day 25 which was 10 days after the elicitation (Figure 5.7). The HPLC profile was shown in Appendix H. This result showed 47-fold increase compared to untreated control collected at the same day. When compared to ZER content collected from *in vivo* rhizome, the increase was 1.5-fold. This showed that the results obtained were comparable to ZER content extracted from rhizome of field grown plant.

For day 20 supplementation time, the maximum ZER production (39130 μg/g DW) was obtained five days after elicitor treatment (Figure 5.7). The HPLC profile was shown in Appendix I. Both supplementations at exponential phase produced significantly high ZER titres. This was in accordance with study conducted by Wang et al. (2015) who observed that MeJA supplementation at exponential phase (day 15 and 20) for cell suspension culture *Hypericum perforatum* significantly increased the flavonoid content and production compared to untreated control. A similar observation was obtained by Huang and Zhong (2013) on *Panax ginseng* cell cultures which showed that elicitation treatment during the log phase reduced the biomass dry weight but enhanced ginsenoside accumulation. From the results, there was a significant decrease observed in ZER production from day 26 to day 30 and the amount of the compound was decrease drastically after day 32 onwards. This huge decrease was attributed to the stationary phase in cells growth and thus production of ZER was simultaneously affected.

For day 25 supplementation time, the ZER production was found to be significantly low for all collected samples in comparison to day 15 and 20 supplementation time. This ineffectiveness was probably caused by the supplementation of MeJA at the late stage of root growth entering the stationary phase. The supplementation of elicitor at a suitable cultivation time should be considered carefully in getting the maximum

compound production. From the study, high expression of ZER was observed approximately between day 24 to day 26 regardless of supplementation time during the exponential phase (day 15 and 20).

The maximum ZER production ensued at approximately similar point of time, which was at the early stationary stage. This was in accordance with a study conducted by Veerashree et al. (2012), who showed high accumulation of gymnemic acid in *Gymnema sylvestre* cell suspensions after 20 days of elicitation with methyl jasmonate. Delayed elicitation (day 25) led to low zerumbone productivity as cell culture was entering the stationary phase.

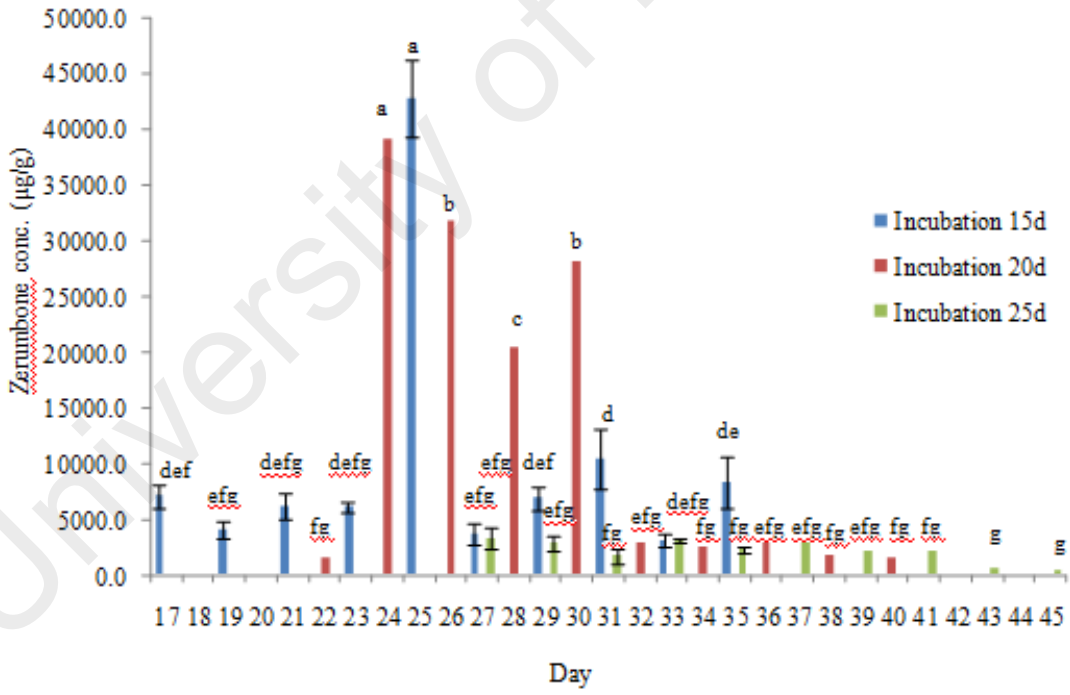

Figure 5.7: The effect of different incubation periods on zerumbone production through MeJA elicitation .

5.3.3 The relationship between zerumbone production and antioxidant enzymes activities.

5.3.3.1 Protein content of adventitious roots

There was evidence that GTP-binding proteins involved in the elicitation of defense response in cultured cells (Legendre et al., 1992). The addition of elicitors was believed to release H_2O_2 that has been proposed to retard pathogen invasion and to stimulate subsequent defense pathways (Bradley et al., 1992) which involved activation of antioxidant enzymes (Sairam et al., 1998). In this study, protein content was determined for control (untreated AdR) and AdR elicited with MeJA at 800 µM. Supplementation of MeJA was carried out at day 15 and samples were collected within a period of 20 days. From the results, the highest protein content (1.44 mg/ml) was obtained on day 19 for untreated AdR, and was significantly higher compared to treated AdR for all sampling times (Figure 5.8). It was concluded that elicitor treatment significantly lowered protein content in AdR of *Z. zerumbet* relative to untreated control. This could support the finding by Legendre et al. (1992) who suggested that GTP-binding proteins participate in some manner in mediating or regulating elicitation of H_2O_2 burst.

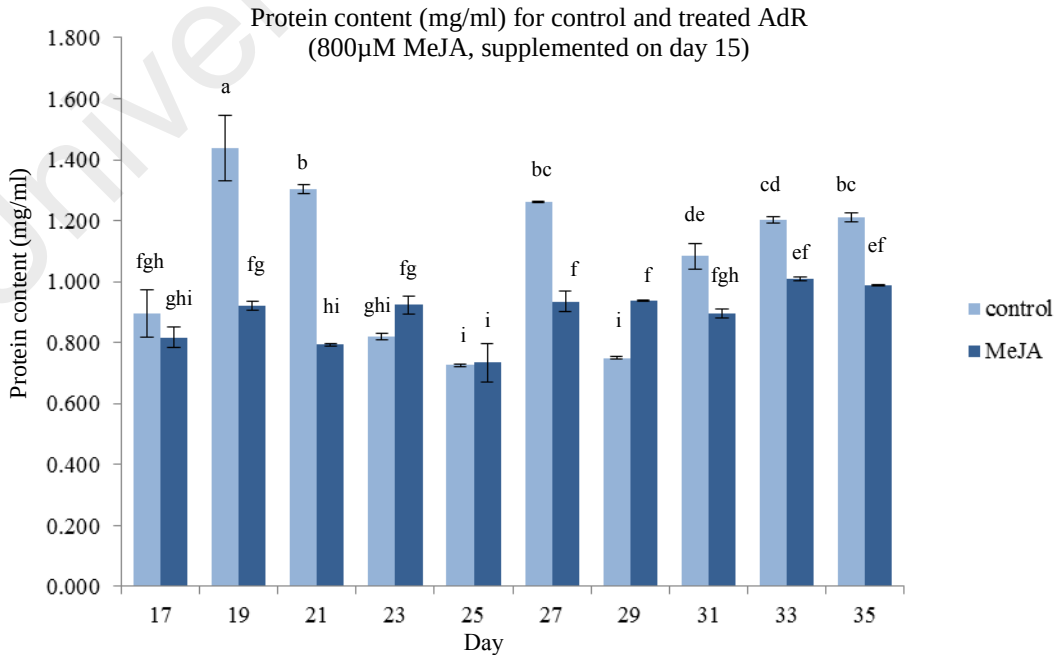

Figure 5.8: Protein content for untreated AdR and AdR elicited with 800 µM MeJA

5.3.3.2 Hydrogen peroxide concentration

Hydrogen peroxide concentration was determined for control (untreated AdR) and AdR elicited with MeJA at 800 µM. Supplementation of MeJA was carried out on day 15 and samples were collected within a period of 20 days. Treatment with elicitor at day 35 showed highest H_2O_2 content (0.124 mM) compared to other sampling times. The results showed that treatment with MeJA co-incided with stress response of the roots culture hence higher H_2O_2 accumulation. From Figure 5.9, it can be observed that the roots reacted to elicitor supplementation after a few days, and highexpression of H_2O_2 content might also be due to other factors involved in cell culture growth stage i.e. stationary phase.

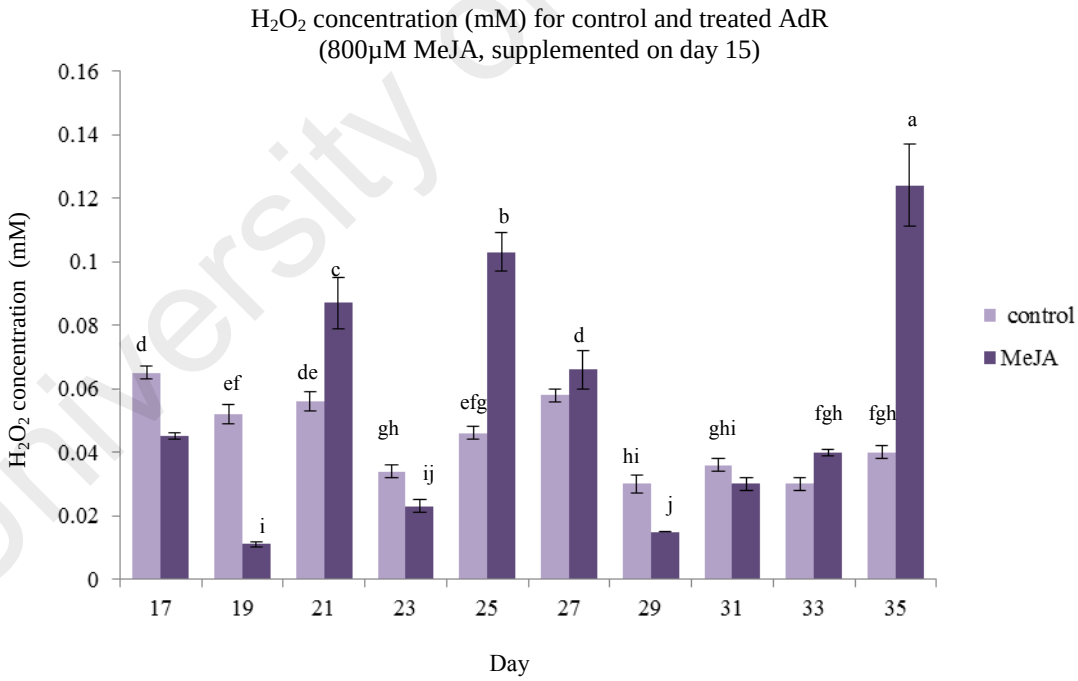

Figure 5.9: Hydrogen peroxide concentrations for control and AdR elicited with 800 µM MeJA.

5.3.3.3 APX specific activity

The APX activity was also determined for control (untreated AdR) and AdR elicited with MeJA at 800 µM. Supplementation of MeJA was carried out on day 15 and samples were collected within a period of 20 days. From the results, it showed that the activity of APX was more pronounced in untreated AdR. The highest APX activity was obtained on day 23 (1.21 U/mg) in untreated AdR (Figure 5.10). The APX specific activity for AdR elicited with 800 µM MeJA showed a significant decrease compared the untreated roots. The low concentration of APX activity was observed even when H_2O_2 was high could be due to the fact that APX cannot convert hydrogen peroxide to water directly. Another contributing factor could be due to high ascorbic acid content that play important role in the scavenging of ROS (Sairam et al., 1998).

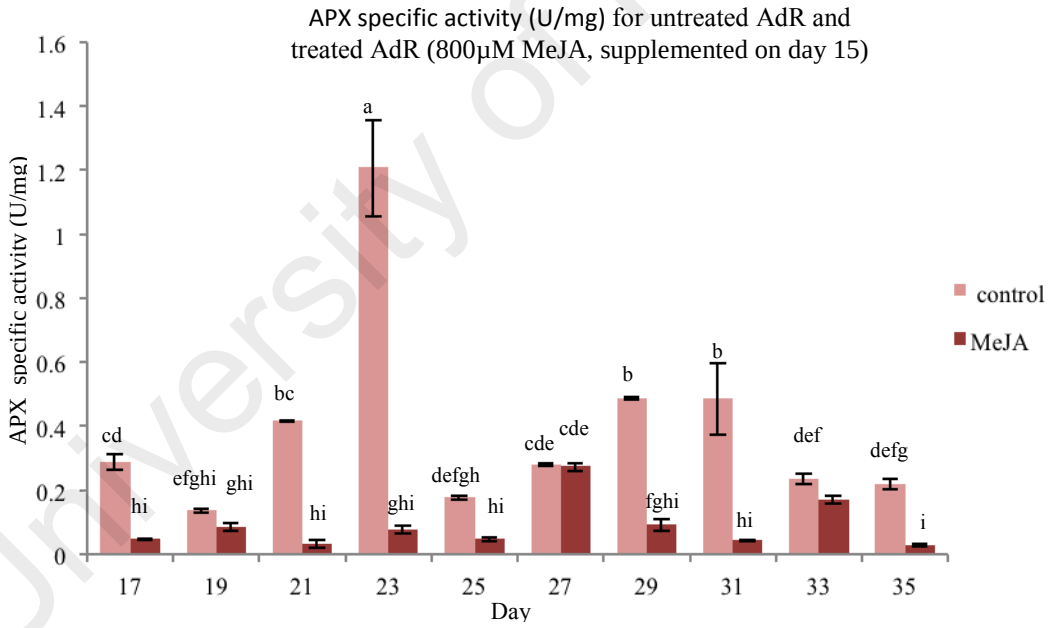

Figure 5.10: APX specific activities for control and AdR elicited with 800 µM MeJA.

5.3.3.4 Catalase specific activity

The activity of CAT was also determined for control (untreated AdR) and AdR elicited with MeJA at 800 µM. Supplementation of MeJA was carried out on day 15 and samples were collected within a period of 20 days. The CAT specific activity was more pronounced for treated AdR with the highest CAT activity observed at day 19 (51.3 U/mg). The activities of the antioxidant enzyme were relatively strong during the early stage following elicitor supplementation and later declined after day 25 (Figure 5.11). The high CAT activities could be related to its scavenging activity towards the reactive oxygen species produced immediately after elicitation process. CAT directly converts H_2O_2 into water and oxygen and, on the contrary of APX, it is more involved in detoxification of H_2O_2 than the regulation as a signaling molecule in plant (Sofo et al., 2015).

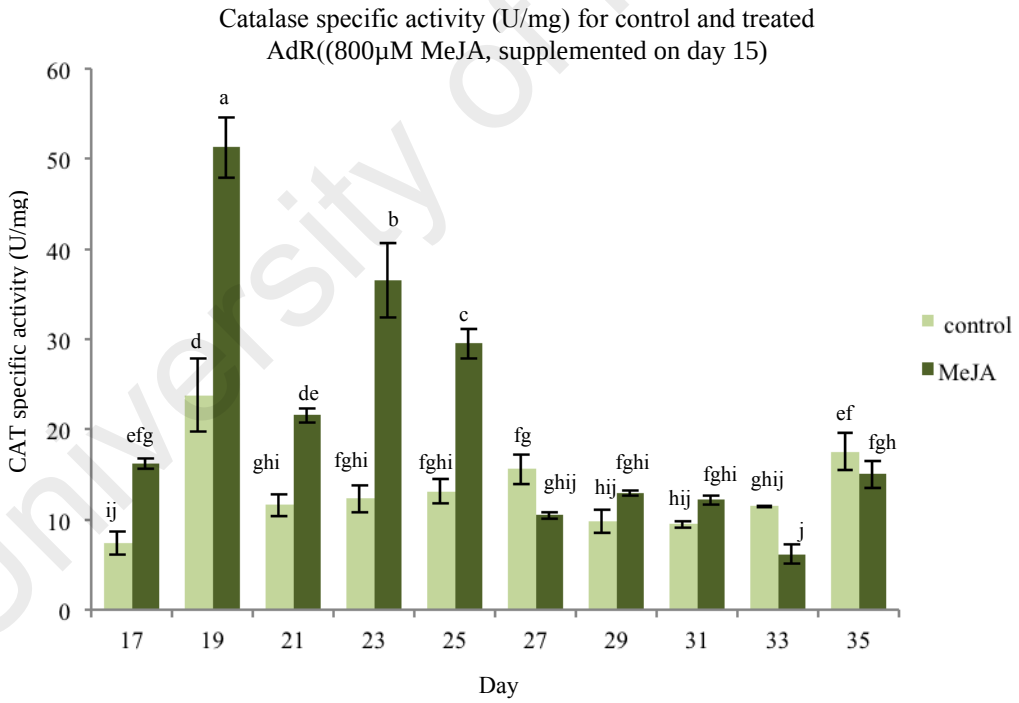

Figure 5.11: CAT specific activities for control and AdR elicited with 800Mm MeJA.

5.3.4 The correlation between zerumbone production with hydrogen peroxide, ascorbate peroxidase (APX) and catalase (CAT) activities following MeJA elicitation of root cultures

The relationship between ZER production and the profile behavior of H_2O_2, APX and CAT was investigated for untreated AdR (control) and AdR elicited with 800 μM MeJA. For control treatment, the ZER production showed an increase up to day 25 but decreased for the next 10 days. For the highest ZER production in control treatment, the concentration of H_2O_2 was higher than the activities of APX and CAT (Figure 5.12). In general, distinct pattern relating the relationship among those variables was absent in the untreated control culture. From the correlation study, the ZER production was significantly affected by the addition of elicitor and collection period (Appendix J).

For MeJA-elicited AdR, it can be observed that the concentration of ZER produced showed significant difference throughout the culture period. Supplementation with MeJA could lead to oxidative stress to the plant cells and induce the generation of various reactive oxygen species (ROS). To avoid damage caused by the excess ROS, plants have developed a biological defense mechanism to scavenge ROS by producing antioxidant enzymes such as catalyse (CAT), ascorbate peroxidase (APX) and superoxide dismutases (SOD) and also non-enzymatic small antioxidant molecules such as glutathione which play important roles in lowering the ROS level and helping to curb the effects of oxidative stress (Sharma et al., 2012).

The highest ZER production was also achieved at day 25, which was 10 days after the elicitor addition (Figure 5.13). At the same, the concentration of H_2O_2 was relatively high in the elicited culture when compared to the control attributed to oxidative stress response of the culture following elicitor addition. Based on the correlation study, the effect of elicitor on H_2O_2 evolution was insignificant. But as time progress, there was significant effect on H_2O_2 evolution (Appendix J). It was also observed that the APX activity was significantly lower in the elicited culture, relative to the control culture,

when ZER production was maximum (Figure 5.13). On day 27, APX was the highest in the elicited culture which co-incided with the very low zerumbone titre. After day 27 onwards, APX activities showed irregular pattern while the ZER titres within this period remained relatively low. The APX activity was significantly affected by the inclusion of elicitor and collection period (Appendix J).

It was also found that CAT activities were relatively higher in elicited culture, when ZER titre was at its maximum (Figure 5.13). This was followed by a decline in its activities after day 25 in the elicited culture, and the activities remained low for the remaining of the culture period co-inciding with low concentrations of ZER from day 27 onwards (Figure 5.13). This also showed that the CAT activity was significantly affected by the inclusion of elicitor and collection period (Appendix J).

While it was clear that discernible pattern of occurrences among the studied variables can be deduced, it is suggested that other detailed and more specific approaches such as proteomic and metabolomic methods to be applied in order to further clarify the roles and regulations of the oxidative stress molecules and enzymes responses in MeJA-elicited AdR cultures of *Z. zerumbet* used in this study. Hong et al. (2013a, b) mentioned of the linked between oxidative stress and secondary metabolite which involve transcriptional regulations.

The occurrence of the sesquiterpenoids in MeJA-elicited cells and other tentatively identified metabolites (abscisic acid, fatty acids, phytosterols and metabolites of shikimate-phenylpropanoid pathways) indicates that the changes in the metabolome are

correlated with a defensive function in response to MeJA elicitation, rather than just the amplification of existing terpene pathways (Tugizimana et al., 2015).

In conclusion, the type and concentration of elicitors are important in optimizing the elicitation of desired compound production without negatively affecting biomass production. An equally important aspect to be taken into consideration is the elicitor supplementation time. The optimized conditions could help to minimize biomass decline and maximize bioactive compound expression.

CHAPTER 6: ESTABLISHMENT OF ADVENTITIOUS ROOT CULTURE AND ZERUMBONE PRODUCTION IN BALLOON-TYPE BUBBLE COLUMN BIOREACTOR (BTBCB)

6.1 INTRODUCTION

The advancement in controlled, bioreactor-based cultivation methods has made plant cell or root suspension cultures capable of producing desired phytomedicinal compounds at a rate comparable or superior to the naturally grown plants. However, to fulfill the demand of increasing population, the major challenge is on how to adopt these technologies for large scale production of bioactive compounds from plant cells or roots culture that are reproducible, safe, and economically viable (Baque et al., 2012b). In this regard, application of bioreactor technology is expected to fulfill the demand for commercial production of bioactive compounds by plant-based systems.

Zingiber zerumbet adventitious root culture system exhibited rapid growth and potentially high zerumbone (ZER) biosynthesis. This opens up an alternative route for an exploitation of compound harvesting through up-scaled culture of the roots in bioreactor. By controlling the micro-environment in bioreactor system, a high degree of control is possible for better handling of the plant culture through optimized aeration, pH, dissolved oxygen, medium circulation, and filtration of the medium. In this chapter, we describe the optimization of adventitious roots cultivation in balloon type bubble column bioreactor (BTBCB) with the aim to study its growth and ZER production in bioreactor.

6.1.1 Types of bioreactor

A bioreactor is a vessel wherein a biological reaction or change in a process occurs. There are several types of bioreactor used in cell culture namely stirred-tank, rotary drum tank, pneumatically driven, bubble column and air-lift bioreactor. For a specific

bioprocess, it is important to have a rational consideration of many factors, including oxygen transfer, shear, mixing, cost, scale-up and operational stability and reliability (Zhong et al., 2010). Bioreactor characteristics and operational mode also strongly affect the biological performance.

For plant cells culture, bubble column and airlift bioreactor are usually preferred compared to other type of bioreactors (Paek et al., 2005). The two types of bioreactors are commercially successful for adventitious root and cell suspension culture due to low shear stress, easy scale-up, low operating and maintenance cost (Hu et al., 2001; Paek et al., 2005; Zhong, 2002). Nonetheless, the major disadvantages of the above-mentioned bioreactors are the formation of foam induced by large volumes of air, and growth of cells in the headspace. The phenomenon of foaming and cell growth on the wall of the vessel is due to similar diameter between the vessel body and its top, and this can be overcame by fabricating a reactor with non-conventional configuration known as balloon column bioreactor (Paek et al., 2001).

The type of culture system in bioreactors can be distinguished into three main classes including biomass-producing culture system (cells or organogenic or embryogenic propagules, shoots or roots as the final product), metabolites and enzymes-producing culture system and biotransformation of exogenously added metabolites culture system which may be precursors in a metabolic pathway (Paek et al., 2005).

6.1.2 Biomass scale up and bioactive compound harvesting from bioreactor

The up-scaling process in bioreactor is mainly focusing on biomass production such as plantlets and somatic embryos multiplication or other organ culture propagation. In previous studies, researchers had successfully cultivated somatic embryos (Gorret et al.,

2004, Shohael et al., 2014), shoot and buds (Escalona et al., 1999; Roels et al., 2005) and plantlets (Piao et al., 2003) in bioreactor systems. With recent development in plant cell cultures, propagation of cells for bioactive compound harvesting is increasing due to demand in pharmaceutical industries. Thus, optimization of different process parameters becomes key challenge towards successful commercial scale-up of plant cell cultures.

In order to achieve production for commercial scale, several key few factors need consideration such as dissolved oxygen, inoculum density, pH, aeration volume and nutrient composition. Baque et al. (2012b) stated that the appropriate optimal nutrients concentrations and their environmental factors are consequential in accelerating the yield and productivity of desired metabolites in plant cell cultures. Presently, among hundreds of secondary plant products that have been investigated, shikonin, ginsenosides and berberine are produced on a large scale and indeed these are the successful stories of an industrial scale-up of plant cell culture applying bioreactor technology (Paek et al., 2005).

Although undifferentiated cell cultures are mainly being studied, a large interest has been shown in adventitious and hairy root cultures. The major problem associated with root culture in bioreactor is the restriction of gaseous oxygen delivery to the central mass of tissue, which resulted in a pocket of senescent tissue. This is due to the root branching which form an interlocked matrix that exhibits a resistance to flow. Nonetheless, the problem could be circumvented through optimization of key cultivation parameters in a bioreactor. Compared to hairy roots culture, adventitious roots (AdR) is free from opine-like toxic compound, thus AdR is preferable for large scale bioactive compound harvesting. Successful adventitious root culture scaling-up

was achieved in ginseng cultivation using 500 L balloon type bubble bioreactor (BTBB) by Choi et al. (2000), and in 10,000 L BTBB by Paek et al. (2009). This is a promising technology that could be applied to Malaysian local plants varieties, which are rich in bioactive compounds to be scaled up to commercial level.

6.2 MATERIALS AND METHODS

6.2.1 Adventitious root cultures

All AdR cultures were derived from the same source of the established roots as described in Chapter 5, section 5.2.1.

6.2.2 Reactor cultivations

To study the growth of AdR in scaled up environment, the AdR obtained from the shake flask was cultured in a five-liters (5 L) balloon type bubble column bioreactor (BTBCB) fabricated by Fermentec Resources Sdn. Bhd, Malaysia (Figure 6.1). The working volume was one-liter and the roots were cultivated by using an optimized media according to Chapter 5, section 5.2.1. One-month old AdR were transferred for into the BTBCB for biomass production and after 30 days of cultivation, roots were harvested for ZER content determination.

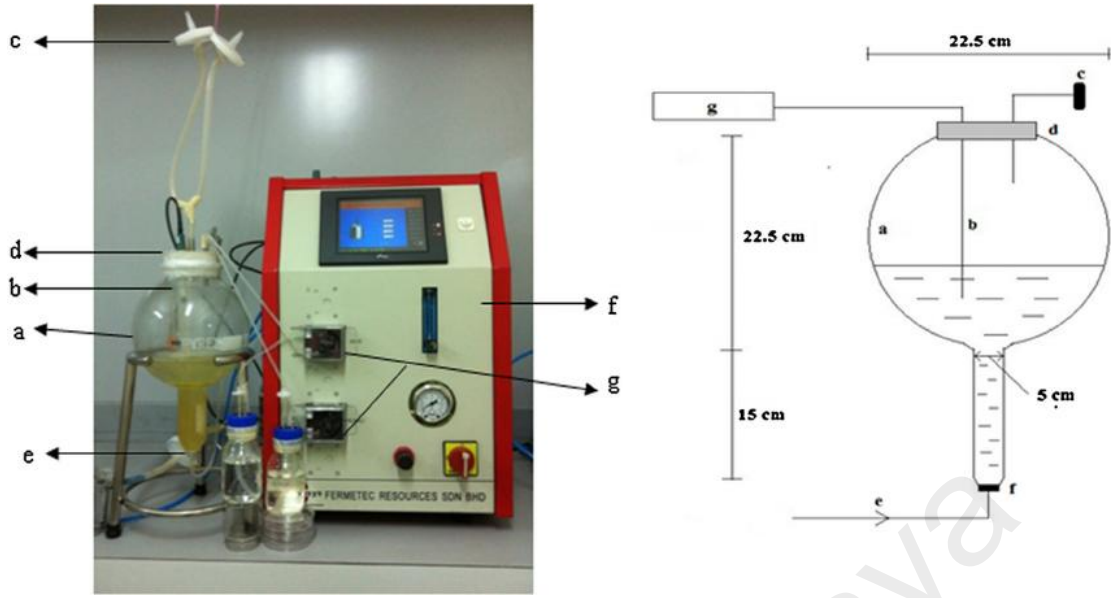

Figure 6.1: Configuration of (a) 5-L balloon type bubble column bioreactor (BTBCB), (b) pH meter, (c) filter air vent, (d) silicon cap, (e) filtered air inlet (f) gas flowrate float meter and (g) peristaltic pump for acid/base addition. Source: Chin *et al*., 2014.

6.2.3 Up-scale production of adventitious roots in bioreactor

6.2.3.1 Inoculum density

The effects of initial inoculum density were observed at four different initial inoculum densities *viz*. 5, 10, 20 and 30g fresh biomass weight (FW). This study was carried out in 1 L working volume of BTBCB at 0.5 L/min air-flow at 25 ± 1°C and pH 5.7. The FW was observed at a five-day interval up to day 30. Growth profile was plotted and final dry weight (DW) was recorded. Based on the growth development, the most productive initial inoculum density was determined. All experiments were carried out in triplicate cultures and were repeated twice.

6.2.3.2 Air flowrate

The effect of air flowrate was observed at five different airflows at 0.2, 0.4, 0.6, 0.8 and 1.0 L/min. This study was carried out in 1 L working volume of BTBCB with initial inoculum at 10g FW and pH 5.7 at 25 ± 0°C. The FW was observed at a five-day interval up to day 30. Growth profile was plotted and final DW was recorded. Based on

the growth development, air flowrate that supported good culture growth was determined. All experiments were carried out in triplicate cultures and were repeated twice.

6.2.3.3 pH

The effect of constant pH was determined at three different pHs *viz.*5.2, 5.7 and 6.2. The pH of the medium was automatically controlled using an automated controller fabricated by Fermetec Resources Sdn. Bhd, Malaysia (Figure 6.1). The pH was regulated by an automatic addition of 0.1 M NaOH or 0.1 M HCL throughout the culture period. This study was carried out in the reactor at 1L/min air flowrate with initial inoculum volume of 10g FW of roots. The fresh biomass was observed at a five-day interval up to day 30. Growth profile was plotted and final DW was recorded. Based on the growth development, the optimum pH that supported good culture growth was determined. All experiments were carried out in triplicate cultures and were repeated twice.

6.2.4 Determination of root biomass

The roots were harvested at day 30 of cultivation for determination of DW. Determination of roots biomass was according to section 4.2.4.4, Chapter 4. The growth data were presented as FW (g/L) and DW (g). The two growth parameters were used for analyzing the biomass growth for AdR cultivated in BTBCB.

$$\text{Fold of biomass produced} = \frac{\text{Final DW} - \text{Initial DW}}{\text{Initial DW}}$$

Maximum growth rate (μ_{max})(day) = $\frac{(\ln X_2 - \ln X_1)}{t_2 - t_1}$

where X_1 is FW at the early log phase and X_2 represents the FW at the end of log phase.

6.2.5 Extraction and HPLC analysis

Root biomass harvested on day 30 was use for ZER content determination. The preparation of extract for ZER analysis was done based on procedure in 4.2.6.2, Chapter 4. The identification of ZER compound was done according to procedure in section 3.2.9, Chapter 3.

6.2.6 Statistical analysis

All data collected were analyzed by one way ANOVA followed by Tukey's test at a significant level of $p = 0.05$.

6.3 RESULTS AND DISCUSSION

6.3.1 Effects of initial inoculum density on biomass growth and zerumbone production

Among the important factors that to be optimized in bioreactor cultivation is the initial inoculum density. Inoculum density affects the performance of plant cells, tissues and organ cultures. This variable could alter the accumulation of biomass and the production of bioactive compounds in *in vitro* cultures (Cui et al. 2014; Jeong et al. 2009).

The AdR of *Z. zerumbet* was successfully cultivated in BTBCB, and more importantly it showed similar morphology to those cultured in shake flask system (Figure 6.2). In this study, highest specific growth rate was achieved at 10 g inoculum

density [5.56 ± 0.31 (× 10^{-2}) day^{-1}] and highest growth ratio at 2.56. Increasing the inoculum volume to 20 and 30 g led to an increased in final FW and DW but showed a reduction in roots growth (Table 6.1 and Figure 6.3). This concurred with the findings of Shohael et al. (2014) that revealed a decline in biomass growth rate when cultured at high initial inoculum. The researchers stated that maximal biomass (11.5 g dry biomass L^{-1}) and eleutheroside production was reached when 5 g cells of inoculum were inoculated into the bioreactors. Inoculation with higher inoculum density (7 and 9 g l^{-1}) was suggested to be responsible for decreasing the accumulated biomass and eleutherosides. However, this was in contrast to Baque et al. (2012b) that stated cell suspension cultures are to be initiated using relatively high inoculum density since a minimum inoculation density resulted in no occurrence of cells growth or is preceded by a lag phase. The actual value of this minimum inoculum size depends on the cell line, nutrient composition of the medium and other culture conditions.

In terms of bioactive compound accumulation, 20 gL^{-1} inoculum volume showed the highest zerumbone production at 900 ± 50 µg/g DW ($p< 0.05$). Decreasing the inoculum density to 5 g L^{-1} showed significantly less ZER production (Figure 6.4). However, when the initial inoculum was at 10- or 30 g L^{-1}, no significant difference was observed in ZER accumulation (Figure 6.4). This was in accordance with findings by Sakurai et al. (1996) working on strawberry cell suspension cultures which indicated that the cell growth and the biosynthesis of anthocyanin were significantly affected by inoculum density. In contrast to this study, Wu et al. (2006) stated that a high inoculum stimulated root growth, but inhibited biosynthesis of phenolics and flavonoids, in adventitious root cultures of *E. angustifolia*. Similarly, in *S. parviflora*, a high inoculum density was found to inhibit scopolamine production (Min et al., 2007). This showed

that an optimized inoculum density plays an important role in both biomass accumulation and compound production.

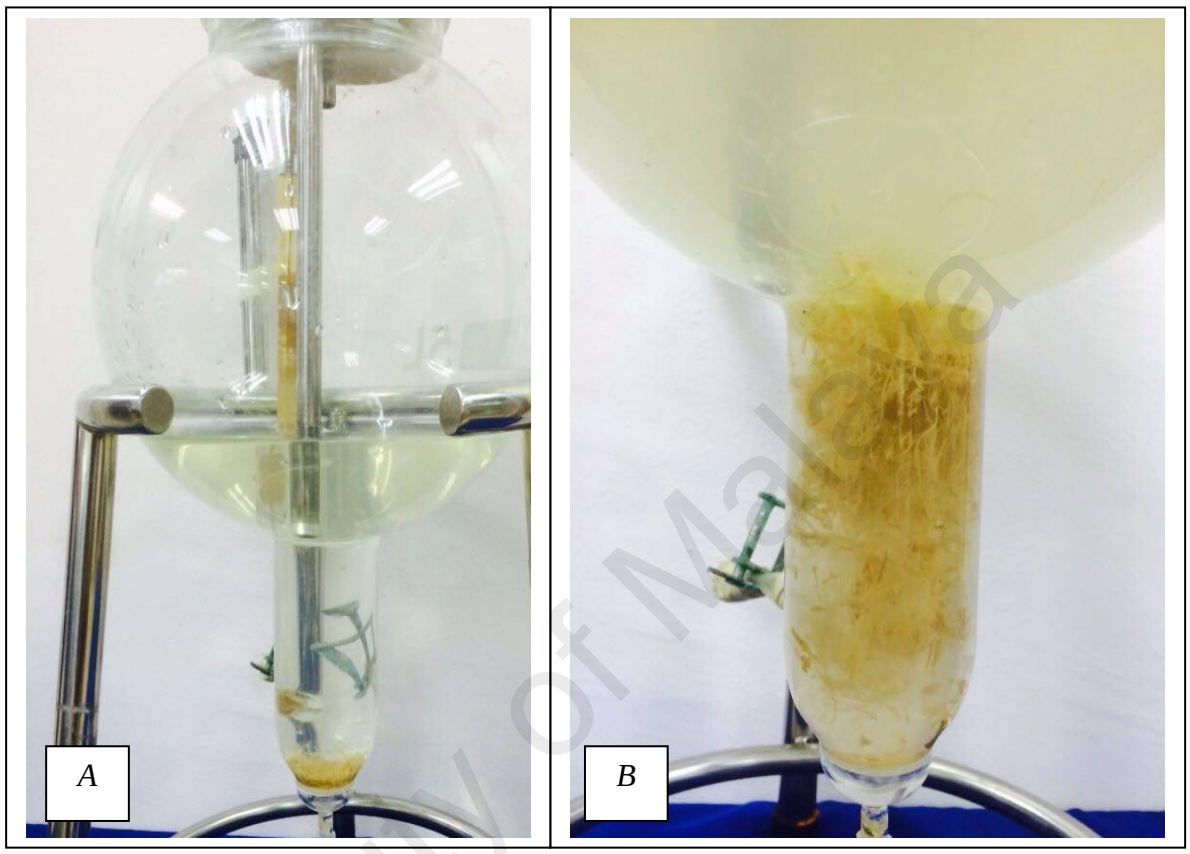

Figure 6.2: Establishment of adventitious root cultures in 5L BTBCB. *A* : roots at day 0 of inoculation. *B* : roots after 25 days of inoculation.

Table 6.1: Comparative values of growth parameters for different initial inocula of *Z. zerumbet* adventitious roots culture in BTBCB.

Inoculum density ($g\ L^{-1}$)	Biomass ($g\ L^{-1}$)		Specific growth rate, μ_{max} ($\times 10^{-2}\ day^{-1}$)	Growth ratio
	Fresh weight	Dry weight		
5	9.0 ± 2.0d	0.92 ± 0.20c	3.0 ± 0.0[d]	0.75
10	34.0 ± 1.0c	3.91 ± 0.60b	5.6 ± 0.3[a]	2.56
20	59.3 ± 4.0b	5.82 ± 0.59a	4.3 ± 0.1[b]	1.43
30	70.7 ± 2.1a	6.20 ±0.63a	3.7 ± 0.1[c]	1.26

Error bar indicates the standard deviation of mean values (*n*=3)

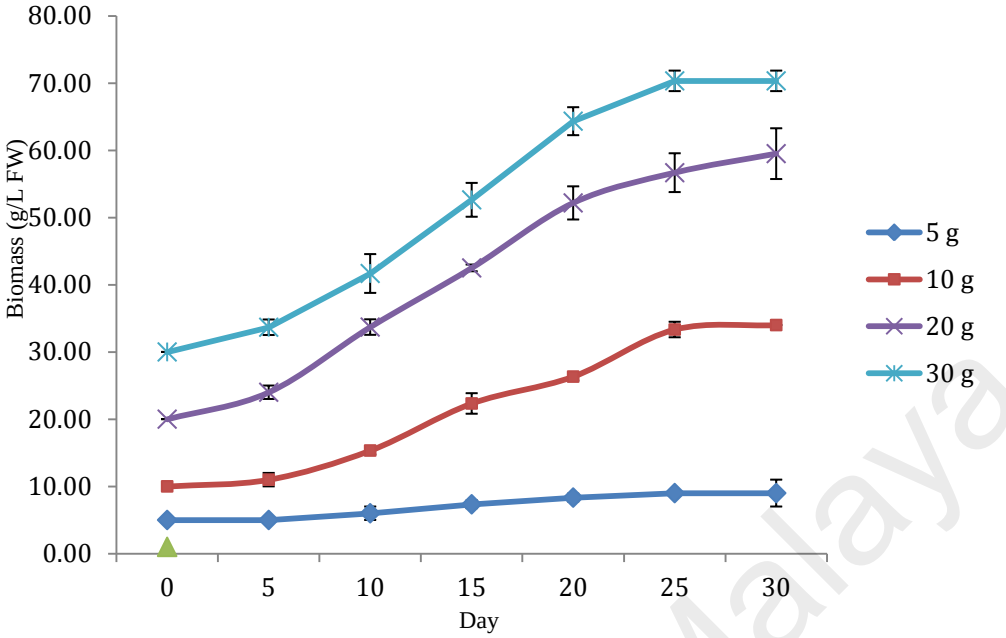

Figure 6.3: The effect of inoculum volume on growth profile of AdR biomass of *Zingiber zerumbet* in BTBCB. Error bars indicate the standard deviation of the mean values.

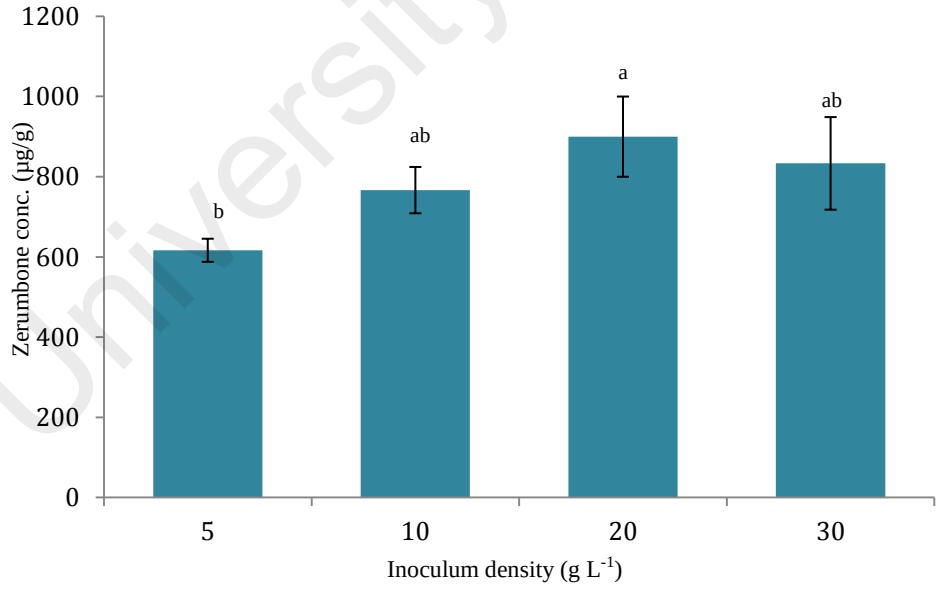

Figure 6.4: The effect of initial inoculum on zerumbone production in AdR of *Z. zerumbet* cultivated in BTBCB. Error bars indicate the standard deviation of the mean values.

6.3.2 Effects of air flowrate on biomass growth and zerumbone production

Gaseous oxygen in the form of continuously supplied air to the bioreactor determines the degree of aeration and agitation in addition to prevent biomass settling, consequently affecting the growth and proliferation of cell culture. Adequate aeration also ensured a continuous oxygen supply to the cells cultivated in bioreactor. Oxygen requirement varies from species to species and it has an effect on metabolic activity and energy supply to the plant cell (Yesil-Celiktas et al., 2010).

In this study, the effects of air flow rate were observed at five different levels. The biomass data and growth profile were shown in Table 6.2 and Figure 6.5 respectively. From the growth profile, the highest specific growth rate was achieved at 1.0 L/min (6.15 ± 0.19 ($\times 10^{-2}$) day^{-1}) with three-fold increment of initial inoculated biomass. High final FW and DW was also achieved at 1.0 L/min flow rate at three-folds of root growth within a month of cultivation. Decreasing air flowrate caused to lower growth of AdR. This could be due to poor fluid mixing and significant oxygen gradient inside the reactor vessel causing less than satisfactory growth of AdR. Aeration is an important factor that promotes better growth by accelerating the transfer of nutrients from liquid *via* fluid hydrodynamics and gaseous phases to roots or cells through dispersion of air bubbles for effective oxygenation (Zhong et al., 2002). Shohael and Paek (2013) also stated that to ensure proper amount of oxygen circulation to the growing biomass, higher aeration rates are usually required. This finding was in agreement with Wang et al. (2015b) that observed the growth rate of *P. quinquefolium* L. adventitious roots gradually increased as aeration volume was raised from 0.3 to 0.5 vvm, and then gradually reduced when the aeration was further increased from 0.5 to 0.7 vvm. In the case of single cell culture, the growth ratio of somatic embryos of *Eleutherococcus senticosus* in bioreactors was highest at lower aeration (0.05vvm) and increasing the

aeration resulted in reduced growth ratio (Shohael & Paek, 2013). The difference in aeration rate requirement between somatic embryos and AdR roots culture could be explained based on the differences in their morphological structure. Inadequate mixing may lead to root clumping, thereby complicating the cultivation in the reactor system. Additionally, the inner cells of the clumps become nutrient shortage, which may have either an adverse or a positive impact on the cell growth and product formation (Panda et al., 1989).

Optimum aeration is not only important factor for growth but as well as secondary metabolites production. In this study, it was observed that highest ZER accumulation was obtained at air flowrate most conducive for biomass accumulation i.e. 1.0 min/L, where a titer of 1000 ± 100 µg/g DW was obtained (Figure 6.6). This was in agreement with finding by Shohael and Paek (2013) that chlorogenic acid and phenolic compound accumulation required higher aeration volume in comparison of aeration needed for high biomass accumulation. It was also observed that increasing the aeration volume 0.05/0.1/0.2/0.3 vvm at 10-day intervals, resulted in highest $k_L a$ coefficient. A relatively high aeration volume speeds up the oxygen transfer to the cells in bioreactor culture, thus allowing cells at the core of roots to receive adequate oxygen supply. In general, a sufficient aeration is beneficial in increasing the rate of oxygen transfer into a bioreactor, which improves both cell growth and secondary metabolite accumulation. Finding in this study also strongly supported that oxygen supply is significantly affecting secondary metabolite formation in plant cell cultures as reported by others (Gao & Lee, 1992; Zhong et al., 1993). In conclusion, air flowrate is another key factor in obtaining maximum biomass growth and bioactive compound accumulation.

Table 6.2: Comparative values of growth parameters at different air flowrates of *Z. zerumbet* adventitious roots culture in BTBCB.

Air flow rate (L/min or vvm)	Biomass (g L^{-1})		Specific growth rate, μ_{max} ($\times 10^{-2}$ day^{-1})	Growth ratio
	Fresh weight	Dry weight		
0.2	13.8 ± 0.3^{d}	1.45 ± 0.21^{c}	1.4 ± 0.2^{c}	0.32
0.4	15.7 ± 0.6^{d}	1.63 ± 0.14^{c}	2.1 ± 0.2^{c}	0.48
0.6	19.7 ± 0.6^{c}	$2.16 \pm 0.40b^{c}$	3.0 ± 0.1^{bc}	0.96
0.8	27.8 ± 0.3^{b}	4.06 ± 1.43^{ab}	4.9 ± 0.2^{ab}	2.69
1.0	38.7 ± 2.1^{a}	4.64 ± 0.65^{a}	6.2 ± 0.2^{a}	3.22

Error bar indicates the standard deviation of mean values (n=3)

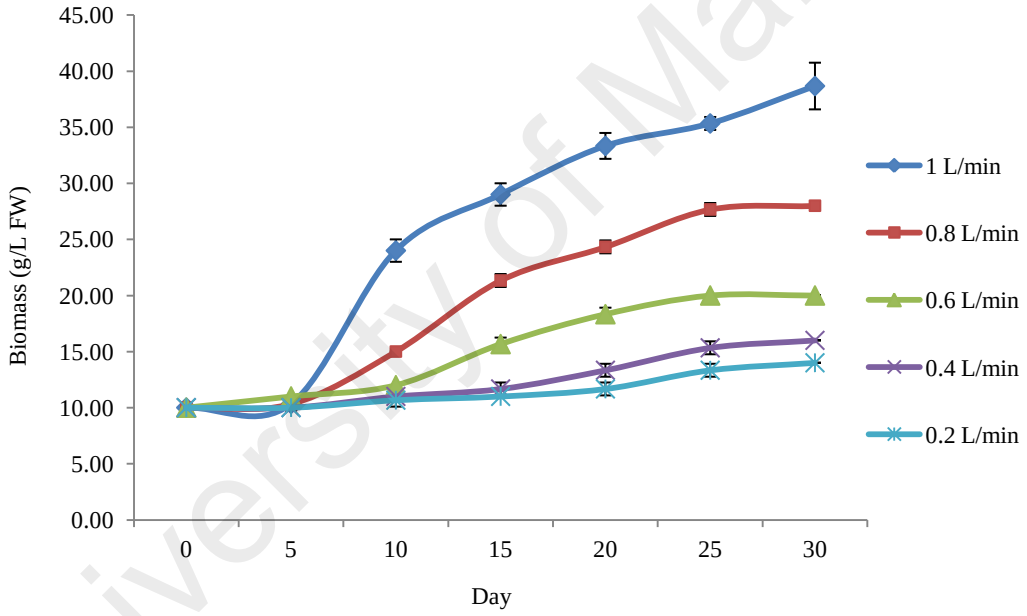

Figure 6.5: The effect of air flowrates on growth profiles of AdR biomass of *Zingiber zerumbet* in BTBCB. Error bars indicate the standard deviation of the mean values.

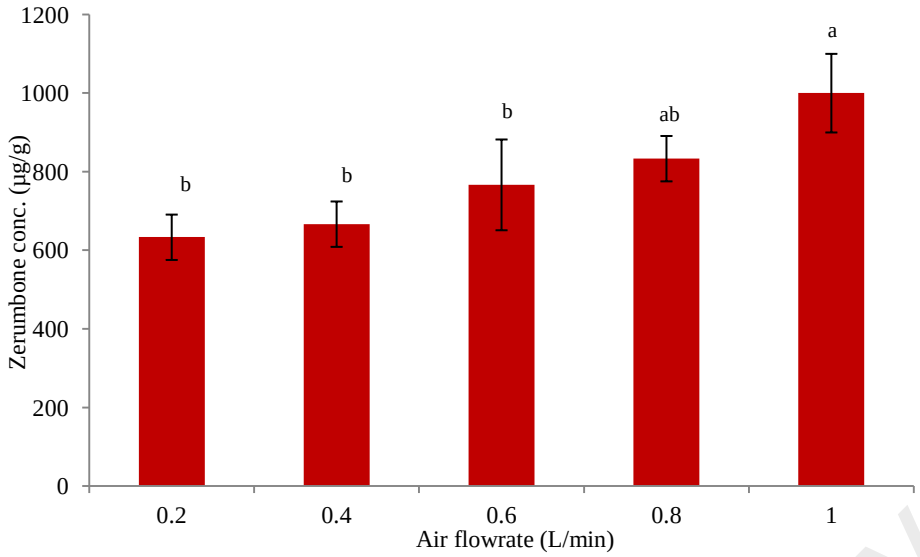

Figure 6.6: The effect of air flowrate on zerumbone production in AdR of *Z. zerumbet* cultivated in BTBCB. Error bars indicate the standard deviation of the mean values.

6.3.3 Effects of pH on biomass growth and zerumbone production

In bioreactor cultivation, optimum growth conditions can be achieved by regulating various chemical and physical factors, including mixing, efficient oxygen transfer, pH, gaseous composition and hydrodynamic forces (Dong et al., 2013). Gorret et al. (2004) emphasized the importance of medium pH for adventitious roots growth, and it varies differently from species to species. The pH control in cell culture is related to nutrient uptake by the cells which is greatly affected by the hydrogen ion concentration of the cultivation medium (George et al., 2008).

In this study, the AdR cultures cultivated in a BTBCB with controlled pH at three levels (pH 5.2, 5.7 and 6.2) showed almost similar growth profiles (Figure 6.7). The highest specific growth rate was achieved at pH 5.7 [5.5 ± 0.3 ($\times 10^{-2}$ day^{-1})] with three-folds of biomass increment from initial volume (Table 6.3). This finding was in accordance with Chin et al. (2014) on cell suspension cultures of banana that reported pH controlled at 5.7 produced higher cell growth rate compared to those grown in

BTBCB system without pH control. Similar observation was obtained for cell suspension culture of *Z. zerumbet* where medium adjusted to initial pH 5.7 recorded the highest specific growth rate (Jalil *et al*., 2015). Jayaraman et al. (2014) also mentioned of the effectiveness of media condition at pH 5.7 in inducing cell suspension culture establishment from friable callus of *Aquilaria malaccensis*. It was also noted in this study that, reducing the pH to 5.2 or increasing it to 6.2 resulted in a reduced root development with lower specific growth rate of 4.25 ± 0.3 ($\times 10^{-2}$ day^{-1}) and 4.7 5.0 ± 0.7 ($\times 10^{-2}$ day^{-1}) respectively. Chung et al. (2006) indicated that cell proliferation can be affected by a strong acidic condition and the best regeneration and cell growth occurred in pH ranging from 5.5–6.0. This could be explained as when pH of the culture medium was fixed at 5.7, most of the salts would be available in a soluble form at this pH allowing for favorable uptake thus promoted fast growth and differentiation.

Kevács et al. (1995) also reported that cell growth of maize cell suspension culture was highly affected by the pH in bioreactor. As for ZER production, the pH values studied did not significantly increased its level (Figure 6.8), and no significant difference was observed for the three pH levels investigated with the highest titre measured at 750 ± 50 µg/g DW. This was attributed to the studied pH values to be within the normal range of favorable cultivation requirements. As a conclusion, controlled pH affects the growth of root biomass but showed negligible effects on ZER production by AdR of *Z. zerumbet*.

Table 6.3: Comparative values of growth parameters in different pHs of *Z. zerumbet* adventitious roots culture in BTBCB.

pH	Biomass (g L^{-1})		Specific growth rate, μ_{max} ($\times 10^{-2}$ day^{-1})	Growth ratio
	Fresh weight	Dry weight		
5.2	24.33 ± 1.15^{c}	2.55 ± 0.30^{b}	4.2 ± 0.3^{b}	1.32
5.7	35.50 ± 1.32^{a}	4.43 ± 0.26^{a}	5.5 ± 0.2^{a}	3.03
6.2	31.67 ± 1.15^{b}	3.41 ± 0.55^{b}	4.7 ± 0.7^{b}	2.10

Error bar indicates the standard deviation of mean values (*n*=3)

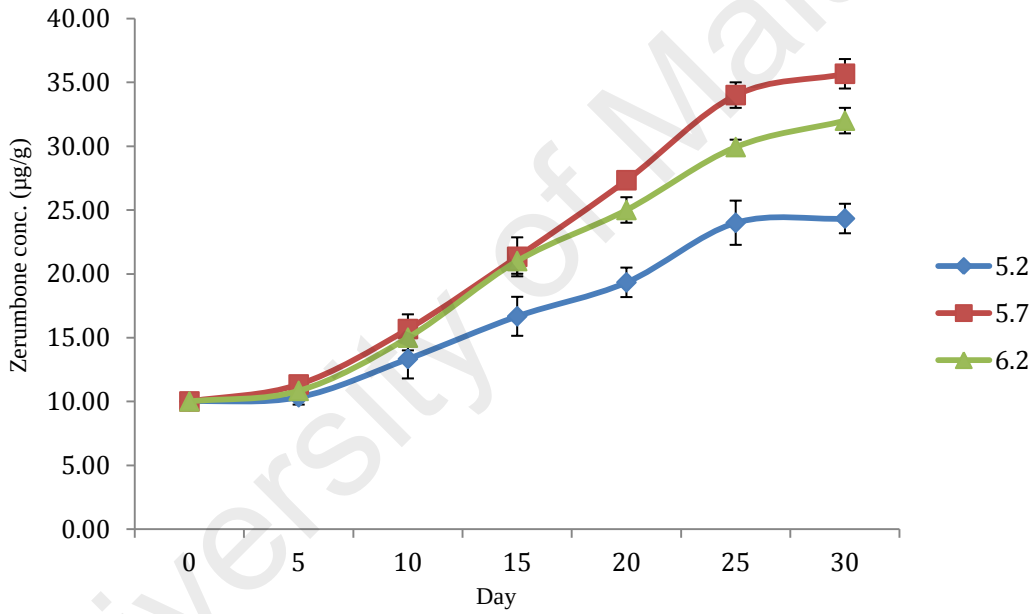

Figure 6.7: The effect of pH on growth profiles of AdR biomass of *Z. zerumbet* in BTBCB. Error bars indicate the standard deviation of the mean values.

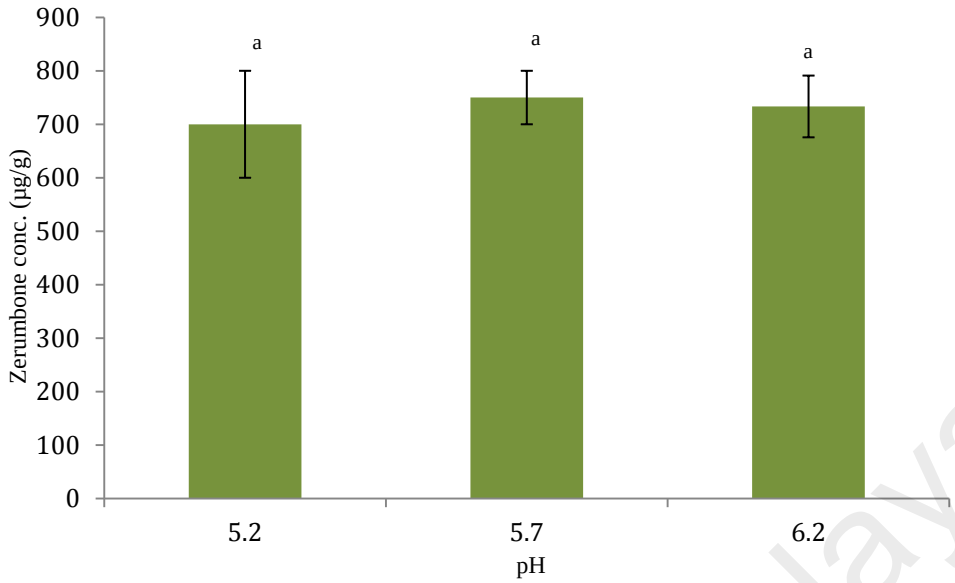

Figure 6.8: The effect of pH on zerumbone production in AdR of *Z. zerumbet* cultivated in BTBCB. Error bars indicate the standard deviation of the mean values.

In this study, it can be concluded that optimization of selected key factors was instrumental in improving root biomass growth and ZER production for bioreactor processes. The optimum cultivation conditions obtained in BTBCB coupled with elicitation strategy could be adopted in order to obtain higher ZER production in controlled bioreactor system.

CHAPTER 7: GENERAL DISCUSSION AND CONCLUSIONS

7.1 GENERAL DISCUSSION

Plant secondary metabolites are a source of potential valuable natural products for pharmaceuticals, flavours and fragrances and fine chemicals (Zhong et al., 1995). *In vitro* propagation of medicinal plants with enriched bioactive compounds along with cell culture methodologies for selective metabolite production is believed to be highly applicable for commercial production of pharmacologically important compounds. In view of this, selected approaches to enhance the yield of bioactive compound i.e. zerumbone in *Zingiber zerumbet* Smith have been investigated and discussed for this study.

For bioactive compound harvesting, a successful protocol was developed to mass propagate cells suspension and adventitious roots of *Z. zerumbet*. This was the first published report of compound harvesting from *in vitro* cells culture of *Z. zerumbet* Smith. *Z. zerumbet* contained highly valued phytomedicinal compound known as zerumbone which showed a versatile pharmacological properties such as anti-atherosclerosis, anti-inflammatory, antimicrobial, antioxidant, anti-AD (Alzheimer's disease), antinociceptive, anti-allergic, anti-edema and involved in hepatoprotective, chemopreventive and cytoprotective activities (Eguchi et al. 2007; Ahmad Bustamam et al., 2008; Taha et al., 2010). Research on *Z. zerumbet* had also demonstrated that zerumbone is a potential drug for the treatment of several cancers as well as leukemia (Kirana et al., 2003; Murakami et al., 2004; Sharifah Sakinah et al., 2007; Xian et al., 2007). Current advances in plant biotechnology provide opportunity to cultivate plant cells, tissues and organs for useful secondary metabolites production instead of whole plant cultivation. In order to obtain materials for compound harvesting, the establishment of cell suspension culture of *Z. zerumbet* was done by

optimizing the growth conditions of the cells through manipulation of parameters such as carbohydrate substrates, sucrose concentrations, pH of medium, light regime, agitation and incubation temperature. In this study, sucrose was found to enhance growth and zerumbone production in cell suspension culture of *Z. zerumbet*. Sucrose is the most important carbon source for development of plant cell culture and biosynthesis of secondary metabolites (Yaseen et al., 2013, Ahmad & Mohammadi, 2016). Sucrose was superior to other carbohydrate sources in cultivation of *Z. zerumbet* cells This was attributed to the fact that when sucrose is used in culture medium, it will be hydrolyzed to glucose and fructose and this helps to increase the osmolality of the medium (Last & Brettell, 1990) while other carbohydrate sources, maltose for instant, was reported to have slow hydrolysis rate thus effecting the biomass growth (Roberts-Oehlschlager et al., 1990).

In this study, it was also noted that the cells cultured in 2 and 3% sucrose showed similar specific growth rates and doubling time while lower sucrose level inhibited the cells growth. In contrast, in determining the best sucrose concentration for zerumbone production, it was found that different levels of sucrose concentration tested did not significantly affect the zerumbone accumulation ($p< 0.05$). The highest specific growth rate of cells was recorded in liquid Murashige and Skoog basal medium containing 3% sucrose with initial pH 5.7 and incubated under continuous shaking condition of 70rpm for 16h light and 8h dark cycle at 24°C. Our results also showed that light regime, agitation speed, and incubation temperature affected the production of zerumbone. Although the zerumbone produced in this study was yet to reach comparable level to *in vivo* rhizome of *Z. zerumbet*, the possibility of producing zerumbone during early stage of cultivation could serve as a platform for subsequent improvement.

In attempt to enhance zerumbone accumulation, adventitious roots initiation was considered. Among various *in vitro* techniques available, the adventitious root culture method is one of the most attractive for producing large amounts of biomass and bioactive compounds due to fairly easy up-scaling process, and bioactive compounds derived from adventitious roots are normally similar to those from their native parental plants (Wang et al., 2013). Several parameters were tested including the application of plant growth hormones namely IBA and NAA in different light regime. In this study, adventitious roots were initiated from newly formed shoot buds of the rhizome through direct and indirect organogenesis. It was found that the frequency of root response, number of roots per explant, root length, and zerumbone production were influenced by the concentrations and the types of auxins used for both adventitious root derived from direct and indirect organogenesis. This was supported by the findings of Huang and Sung (2006) that the specific growth rate and secondary metabolite production are strongly influenced by the culture medium composition.

Determination of appropriate initial inoculum volume and medium strength was further carried out to enhance the root biomass and zerumbone production. For multiplication of adventitious roots in shake flask system, the highest specific growth rate was achieved in adventitious roots derived from indirect organogenesis with an initial inoculum of 0.5 g FW in full strength Murashige and Skoog (MS) medium, whereas the amount of zerumbone in half strength MS was found to be higher than in full strength MS (2520 µg/g) in roots initiated through direct organogenesis. It can be concluded that the both cell growth and zerumbone production behavior were important in the ultimate choice of explant to be used. Our study provided important groundwork for further research on the biosynthetic mechanism and scalable protocol for the production of zerumbone.

In most cases, plant cell or root cultures failed to produce remarkably high amount of targeted product. In order to achieve high productivity, strategies to enhance production of desired metabolites must be considered (Dornenburg & Knorr, 1995; Kim et al., 2005; Jeong et al., 2009). In this study, zerumbone elicitation by using methyl jasmonate and salicylic acid was adopted in adventitious root cultures to enhance its expression to the level comparable to field grown rhizome. The addition of exogenous elicitors has been exploited as effective means for enhancing the production of commercially important metabolites from plant cell or root cultures (Cai et al., 2012). The effectiveness of elicitors in enhancing secondary compound production had been proven by a number of researches (Chodisetti et al., 2015; Hao et al., 2014; Xu et al., 2015). In this work, the signaling compounds methyl jasmonate and salicylic acid were used to enhance the production of zerumbone in *Z. zerumbet* adventitious root culture. A two-stage strategy was used that consisted of growth of biomass for three weeks followed by chemical elicitation to enhance zerumbone production. This system was adopted in a manner where the first stage was the adventitious roots cultivation without elicitor for biomass accumulation followed by potential elicitor addition to enhance metabolites without decreasing root biomass (Baque et al., 2012b). Despite the potentials of elicitation to enhance biosynthesis of bioactive molecules in plant cell culture, researchers have alleged that adventitious root cultures treated with elicitors resulted in strong inhibition of root growth but enhanced bioactive compounds accumulation (Murthy et al., 2008; Paek et al., 2009). In the present study, elicitor treatment enhanced zerumbone expression in *Z. zerumbet* adventitious root culture compared to without the application of elicitors. It was also observed that salicylic acid elicitation was more efficient at lower concentration compared to methyl jasmonate for zerumbone production from adventitious root of *Z. zerumbet*. This was in agreement with the findings by Jeong et al. (2005) that showed ginsenoside production was improved by salicylic elicitation in *Panax ginseng* hairy roots

culture at a lower concentration. Treatment with methyl jasmonate was more effective at higher concentration, while lower concentration of methyl jasmonate was insufficient to elicit the response in *Z. zerumbet* root cultures ($p<0.05$). Even though salicylic acid was superior to methyl jasmonate in eliciting the zerumbone production, the biomass harvested for salicylic acid-treated roots was significantly lower to methyl jasmonate-treated culture thus necessitating longer period of cultivation prior to harvesting. It was suggested that salicylic acid negatively affected the biomass production due to inhibition of growth by the signalling transducer when compared methyl jasmonate elicitation (Lee et al., 2015). The type of elicitor, time of supplementation and culture duration needs to be taken into account in the optimization of elicitation responses for compound harvesting.

The elicitor supplementation time is one of the key factors that affect cell growth and product yield for plant cell culture. Therefore, in order to enhance and optimize the zerumbone production, the supplementation time of elicitors was studied. Based on the growth profile of adventitious roots, methyl jasmonate supplementation was carried out at different pre-determined times during exponential and early stationary stages. Based on the results, methyl jasmonate treatment during exponential phase (day 15 and 20) led to significant increase in zerumbone expression with significantly high zerumbone production at 42777 μg/g dry weight for supplementation at day 15. This result showed 47-fold and 1.5-fold increase compared to untreated control and *in vivo* rhizome respectively. Another important finding of the study was both supplementations at the exponential phase produced significantly high zerumbone titres. This was in agreement with other studies that methyl jasmonate supplementation at exponential phase significantly increased the flavonoid content in cell suspension culture of *Hypericum perforatum* (Wang et al., 2015). It can be concluded that high expression of zerumbone in adventitious roots of *Z. zerumbet* was observed

approximately between day 24 to day 26 regardless of supplementation time during the exponential phase (day 15 and day 20) but early supplementation (day 15) resulted in slightly higher zerumbone accumulation in comparison to the latter.

The application of elicitors into plant cell cultures constitutes an excellent system to enhance the secondary compounds production but at the same time induced stress to the cell. The oxidative stress is a complex chemical and physiological phenomenon that be an adjunct to virtually all biotic stresses in higher plants and manifested as a result of overproduction and accumulation of reactive oxygen species (ROS) (Demidchik, 2015). In order to scavenge the ROS, plants defense mechanism includes both enzymatic and non-enzymatic systems. Therefore, the activities of enzymes related to oxidative stress alleviation often change with respect to the behavior of secondary metabolites accumulation (Xu et al., 2006). In this study, the activities of two important enzymes i.e. catalase and ascorbate peroxidase (CAT and APX) were assayed to investigate methyl jasmonate elicitation effect. The correlation between zerumbone production and the activity profiles of H_2O_2, APX and CAT were studied for untreated adventitious root cultures (control) and adventitious root cultures elicited with 800 μM MeJA. In general, distinct pattern relating the relationship among those variables was absent in the untreated control culture.In elicited roots, the highest zerumbone production was achieved on day 25, which was 10 days after the elicitor supplementation. It was observed that the concentration of H_2O_2 was relatively high in the elicited culture in comparison to the control. This attributed to oxidative stress response following elicitor addition to the root culture. It could be observed that the APX activity was significantly lower but CAT activities were relatively higher, when zerumbone production was maximum in the elicited culture, relative to the control culture. Hong et al. (2013a, b) proposed the link between oxidative stress and secondary metabolites, which involve transcriptional

regulations. Therefore, even though elicitation could help to enhance bioactive compound accumulation, it must be done with cautiously to minimize oxidative stress while improving compound production at the same time.

Plant cell culture is a useful system in the study of biosynthesis of secondary metabolites in large quantities under sterile and controlled environment. This type of cultivation helps to minimize variations that commonly arise in field–grown plants (Docimo et al., 2015). Considering these facts, large-scale cultivation of adventitious root in bioreactor is considered as a promising approach for the secondary metabolites production of pharmaceutical and nutraceutical interest in comparison to other culture systems such as cell or hairy root culture. This study demonstrated that adventitious root cultures of *Z. zerumbet* could be practically propagated in a controlled balloon type bubble column bioreactor (BTBCB). The effects of cultivation parameters such as initial inoculums volume, air flowrate and constant pH control were investigated. Lee et al. (2011) indicated that inoculum size and medium pH were among the parameters that greatly affect cell growth performance for plant cell culture. In this study, the highest specific growth rate was achieved at 10 g inoculum density [5.56 ± 0.31 ($\times 10^{-2}$) day^{-1}]. Inoculum lower or higher than 10 g led to a decrease in *Z. zerumbet* root growth ratio. Zerumbone production was highest at 20 gL^{-1} inoculum density and increasing the initial density to 30 gL^{-1} caused a significant drop in its production. Wang et al. (2013b) discussed that maintaining a constant high rate of aeration is not always advantageous for compounds accumulation by plant cultures, due to generation of excessive shear stress.

In bioreactors, adequate mixing is essential to ensure adequate supply of nutrients and to prevent the accumulation of toxic metabolites, therefore it is a critical parameter to be studied and evaluated (Zhong et al., 2010). In large-scale bioreactors, poor mixing often leads to

undesirable concentration gradients and a decrease in mass transfer efficiency. For plant cell cultures, which are typically sensitive to shear stress, an intense agitation or aeration can bring damage to the cells. Therefore, it is important to find the maximum aeration rate without causing hydrodynamic-induced cell damage to the culture. In order to ensure continuous supplied air to the bioreactor, the degree of aeration needs to be optimized for better growth and proliferation of cell culture. In this study, it was found that the highest specific growth rate was achieved at 1.0 L/min (6.15 ± 0.19 ($\times 10^{-2}$) day^{-1}) with three-fold increment of initial inoculated biomass. Decreasing air flowrate caused lower roots growth due to poor fluid mixing that led to low oxygen supply. In this study, it was observed that highest zerumbone production was obtained at air flowrate most conducive for biomass accumulation i.e. 1.0 min/L, where a titre of 1000 ± 100 µg/g DW was measured. This finding was in agreement with Shohael and Paek (2013) who observed good bioactive compound yield normally requires sufficient aeration rate. It can be concluded that relatively high aeration rate helps to increase the oxygen transfer rate to the cells in bioreactor culture thus allowing cells at the core of roots to receive adequate oxygen supply, which improves both secondary metabolite accumulation and cell growth. Another parameter that needs to be considered during up-scaling process of plant cell culture is the constant pH during cultivation. The pH control in cell culture is related to nutrient uptake by the cells which is greatly affected by the hydrogen ion concentration of the cultivation medium (George et al., 2008). From this work, it was observed that the pH affected biomass growth but did not significantly affect zerumbone production in adventitious root cultures of *Z. zerumbet*. This was attributed to the normal range of pH studied which encouraged root growth but was unable to cause further stimulation of bioactive compound production. As a conclusion, optimization of selected key factors during up-scaling of root culture was important in order

to improve biomass accumulation and at the same time enhancing the bioactive compound production cultivated in BTBCB.

In summary, the results from this study provided an essential platform for zerumbone production at a larger scale later. The adventitious root culture system can be used as a model system to provide consistent, reproducible and sustainable materials for various in-depth studies such as metabolomics, proteomics and transcriptomics. Through proteomics and transcriptomics studies, the identified genes could be exploited by down regulating or over expressing them to enhance targeted compounds in *Z. zerumbet* root cultures. Advanced fundamental study is also important in understanding the relationship between oxidative stresses in plant defense mechanism with regards to exogenous elicitor supplementation.

7.2 CONCLUSIONS

In conclusion, this study has successfully established cell suspension and adventitious root cultures of *Z. zerumbet* for biomass and zerumbone production. The productivity of biomass growth and zerumbone accumulation was found to be dependent on established physicochemical parameters for cell suspension cultures. Growth of root biomass was found to be dependent upon the volume of inoculum, type of plant growth regulator applied and light regime. Optimization of root culture in shake flasks and its subsequent up-scaling of in controlled bioreactor were successful. The study also showed that elicitation with methyl jasmonate and salicylic acid increased the zerumbone production, and optimization of elicitor supplementation in terms of supplied concentration and addition time was critical to achieve high zerumbone production at a level comparable to field grown rhizome. Consequently, future strategies could be developed to bring *in vitro* zerumbone production to the path of commercial success.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

1. Jalil, M., Annuar, M. S. M, Tan, B. C. & Khalid, N. (2015). Effect of selected physicochemical parameters on zerumbone production of *Zingiber zerumbet* Smith cell suspension culture. *Evident-Based Complementary and Alternative Medicine*, Vol. 2015, Article ID 757514, 7 pages.

2. Jalil, M., Annuar, M. S. M, Tan, B. C. & Khalid, N. Establishment of *Zingiber zerumbet* Smith cell suspension culture and zerumbone production: chemical and physical factors. *International Conference on Natural Product 2014*, 18-19 March 2014, Palm Garden Hotel, Putrajaya (International Level).

3. Jalil, M., Alwakil, N.H., Tan, B. C., Annuar, M. S. M, & Khalid, N. Establishment of adventitious root cultures through direct and indirect organogenesis in medicinal ginger *Zingiber zerumbet* Smith, *International Conference of Molecular Biology and Biotechnology 2016*, 9-11 March 2016, Connexion@Bangsar South, (International Level).