PRODUCTION OF ALPHA HUMULENE AND ZERUMBONE IN ADVENTITIOUS ROOT CULTURES OF *Zingiber zerumbet* (L.) SMITH THROUGH SYNERGISTIC EFFECT OF ELICITORS AND PLANT GROWTH REGULATORS

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PRODUCTION OF ALPHA HUMULENE AND ZERUMBONE IN ADVENTITIOUS ROOT CULTURES OF *Zingiber zerumbet* (L.) SMITH THROUGH SYNERGISTIC EFFECT OF ELICITORS AND PLANT GROWTH REGULATORS

ABSTRACT

Plant secondary metabolites are gaining huge interest from their phytomedical potential *viz.* antimicrobial, anticancer, anti-inflammatory, drug and pesticides. There are many active researches being carried out to isolate and commercialize these valuable bio-products. Plant cell culture is a feasible alternative route to produce the bioactive compounds as its offers good quality materials less affected by uncertainties in environmental, ecological or climatic conditions. *Zingiber zerumbet* Smith widely known as shampoo ginger or common to Malay as 'Lempoyang' is a small perennial medicinal herb plant belonging to the Zingiberaceae family. Zerumbone presents dominantly in the rhizome of *Zingiber zerumbet* with α-humulene as its intermediate, and exhibits important phytomedicinal properties such as anticancer, antimicrobial, anti-inflammatory, antinociceptive, chemopreventive and antioxidant. Efficient production of zerumbone through the establishment of adventitious root culture has been investigated previously. However, the production of α-humulene and the synergistic effects of elicitors on the production of α-humulene and zerumbone *in vitro* are yet to be investigated. The combination of auxin-auxin and auxin-cytokinin causes a significant decrease in adventitious root growth at high IBA and BAP concentrations. The highest specific growth rate for root biomass production is obtained on media supplemented with 5 mg L^{-1} IBA and 1 mg L^{-1} NAA with maximum fresh weight of 6.90 ± 0.08 g and dry weight of 2.10 ± 0.01 g was not significant with 3 mg L^{-1} BAP and 1 mg L^{-1} NAA. Meanwhile, maximum productions of α-humulene (3759 ± 798 µg/g) and zerumbone (3440 ± 168 µg/g) are observed in the media containing 3 mg L^{-1} BAP and 1 mg L^{-1} NAA under 16:08

photoperiod along with abundant root biomass (1.90 ± 0.05 g DW). Elicitation studies shows that root growth and compounds production are negatively correlated events, which a significance decline in biomass growth is observed with the increase in elicitor concentration. The yields of zerumbone and α-humulene at 43 mg/g DW and 15.8 mg/g DW, respectively are obtained at low concentration range of elicitors i.e. 400μM to 600μM methyl jasmonate and salicyclic acid and represent better production compared to control. In conclusion, synergistic effects from application of elicitor combination to induce excess secondary metabolites in plant culture could be a practical route for larger scale production.

Keywords: Lempoyang; zerumbone; α-humulene; synergistic effect; elicitors.

PRODUCTION OF ALPHA HUMULENE AND ZERUMBONE IN ADVENTITIOUS ROOT CULTURES OF *Zingiber zerumbet* (L.) SMITH THROUGH SYNERGISTIC EFFECT OF ELICITORS AND PLANT GROWTH REGULATORS

ABSTRAK

Metabolit sekunder tumbuhan semakin mendapat perhatian kerana potensi fitomedikalnya seperti antimikrob, antikanser, anti-radang, ubat-ubatan dan racun perosak. Terdapat banyak penyelidikan yang giat dilakukan untuk mengasingkan dan mengkomersialkan bio-produk berharga ini. Kultur sel tumbuhan adalah alternatif yang baik untuk menghasilkan sebatian bioaktif ini kerana ia menawarkan bahan yang berkualiti baik yang kurang dipengaruhi oleh keadaan persekitaran, ekologi atau iklim. *Zingiber zerumbet* Smith dikenali sebagai syampoo halia atau biasanya bagi orang Melayu sebagai 'Lempoyang' adalah tanaman herba tahunan kecil yang tergolong dalam keluarga Zingiberaceae. Zerumbon yang terdapat secara dominan dalam rizom *Zingiber zerumbet* dengan α-humulene sebagai perantaraannya mempunyai pelbagai sifat fitomedikal penting seperti antikanser, antimikrob, anti-keradangan, antinociceptif, kemopreventif dan antoksidan. Penghasilan zerumbon yang cekap melalui penghasilan sistem ampaian akar adventitius telah disiasat sebelum ini. Walau bagaimanapun, penghasilan α-humulene dan kesan sinergistik elisitor terhadap pengeluaran α-humulene dan zerumbon secara *in vitro* masih belum disiasat. Kesan dari gabungan auksin-auksin dan auksin-sitokinin menyebabkan penurunan yang ketara dalam pertumbuhan akar adventitius pada kepekatan IBA dan BAP yang tinggi. Kadar pertumbuhan spesifik tertinggi untuk pengeluaran biojisim akar diperoleh pada media yang ditambah dengan 5 mgL^{-1} IBA dan 1 mgL^{-1} NAA dengan berat segar maksimum 6.90 ± 0.08 g dan berat kering 2.10 ± 0.01 g. Sementara itu, penghasilan maksimum α-humulen (3759 ± 798 μg/g) and zerumbone (3440 ± 168 μg/g) dapat diperhatikan dalam media yang

mengandungi 3 mgL^{-1} BAP dan 1 mgL^{-1} NAA pada dengan pengkalaan cahaya 16 : 8 jam (cahaya : gelap) yang menghasilkan biojisim akar yang banyak (1.90 ± 0.05 g berat kering). Kajian elisitasi menunjukkan bahawa pertumbuhan akar dan pengeluaran sebatian adalah berkorelasi negatif dan juga diperhatikan bahawa pengeluaran biojisim menunjukkan penurunan yang signifikan ketika kepekatan elisitor meningkat. Hasil zerumbone dan α-humulene adalah 43 mg / g DW dan 15.8 mg / g DW, masing-masing diperoleh pada julat kepekatan elisitor yang rendah iaitu 400 μM hingga 600 μM metil jasmonat dan asid salisilik dan mewakili pengeluaran yang lebih baik berbanding dengan kawalan. Sebagai kesimpulan, kesan sinergi dari penggunaan kombinasi elisitor untuk mendorong metabolit sekunder yang berlebihan dalam kultur tumbuhan dapat menjadi jalan praktikal untuk pengeluaran skala yang lebih besar.

Kata kunci: Lempoyang; zerumbon; α-humulen; kesan sinergistik; elisitor.

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viz.	:	Latin : vidalicet or English : namely
et al.	:	Latin : et alii or English : and others
spp.	:	species
ACN	:	acetonitrile
AdRC	:	adventitious root
α	:	alpha
AR (grade)	:	analytical (grade)
BTBCB	:	balloon-type bubble column bioreactor
BA	:	benzyladenine
BAP	:	benzylaminopurine
C	:	carbon
cm	:	centimetre
d	:	day
°C	:	degree celcius
DCM	:	dichloromethane
2,4-D	:	dichlorophenoxyacetic acid
DW	:	dry weight
EDTA	:	ethylenediaminetetra acetic acid
e.g	:	for example
FW	:	fresh weight
GC	:	gas chromatography
GCMS	:	gas chromatography mass spectrometry
g	:	gram

g/L	:	gram per litre
HPLC	:	high performance liquid chromatography
h	:	hour
HCl	:	hydrochloric acid
IBA	:	indole butyric acid
IAA	:	indole-3 acetic acid
JA	:	jasmonic acid
l	:	litre
L/min	:	litre per minute
MeOH	:	methanol
MeJA	:	methyl jasmonate
m	:	metre
µg	:	microgram
µg/g	:	microgram per gram
µl/L	:	microliter per litre
µm	:	micrometre
µM	:	micromolar
µmol	:	micromole
mg	:	milligram
mgL^{-1}	:	milligram per litre
mg/ml	:	milligram per millilitre
ml	:	millilitre
mm	:	millimetre
mM	:	millimolar
min	:	minute
±	:	more less

MS	:	Murashige and Skoog (1962)
nm	:	nanometre
NAA	:	naphthaleneacetic acid
%	:	percentage
PGR	:	plant growth regulator
psi	:	pound per square inch
rpm	:	revolution per minute
SA	:	salicylic acid
s	:	second
SCV	:	settle cell volume
NaCl	:	sodium chloride
NaOH	:	sodium hydroxide
R^2	:	square of the correlation coefficient
SD	:	standard deviation
SE	:	standard error
pH	:	The negative logarithm of the hygrogen ion concentration
t	:	time
uv	:	ultra violets
U	:	unit
v/v	:	volume per volume
vvm	:	volume per volume per minute
λ	:	wavelength
w/v	:	weight per volume

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CHAPTER 1: INTRODUCTION

1.1 Background study

Many modern drugs have been isolated naturally from plant following the observation of their therapeutic efficacies in traditional medicine (Veeresham, 2012). There are many native plant families considered as medicinal plants in Malaysia such as Zingiberaceae, Amaranthaceae, Orchidaceae, Verbenaceae, Caricaceae, Rutaceae, Lamiaceae, Myrtaceae and Clusiaceae. The family of Zingiberaceae are prominent in phytomedicine industry as many species in this family had been shown to exhibit medicinal properties such as *Kaempferia galangal* (Umar *et al.*, 2011), *Curcuma longa* (Labban, 2014), *Zingiber striolatum* (Tian *et al.*, 2020), *Zingiber officinale* (Archana *et al.*, 2013) and *Zingiber zerumbet* (Jalil *et al.*, 2015).

Zingiber zerumbet Smith is a small perennial medicinal herb plant were known as shampoo ginger or called as 'Lempoyang' among native Malay. *Z. zerumbet* consisted of zerumbone (37 %) followed by α-humulene (14 %) and camphene (13.8 %) in the rhizome extract. These compounds have been associated with antioxidant, antiallergic, antihyperglycemic, anti-inflammatory, antimicrobial, antiulcer and anticancer activities. Due to its numerous phytomedicinal values, *Z. zerumbet* rhizome has been extensively cultivated and investigated. The plant is cultivated mainly in Asia and sub-tropical region (Yob *et al.*, 2011). Nonetheless, Nalawade *et al.* (2003) reported that the rhizome can only be stored for a short period due to fungal disease that caused its deterioration.

Furthermore, Gerth *et al.* (2007) indicated that infestation, diseases, application of pesticides and environmental conditions affect the quality and quantity of the secondary

metabolites collected from wild and field grown plants. However, these problems can be circumvented by adopting biotechnological approach known as plant tissue culture.

Over the years, research in plant tissue culture helped to produce phytochemical drugs such as morphine, codeine, reserpine and *L*-DOPA. There are many successful industrial production of plant metabolites using cell cultures have been reported such as *Nicotiana tabacum* (Zhao *et al.*, 2013), *Atropa belladonna* (Yang *et al.*, 2011), *Panax ginseng* (Paek *et al.*, 2009) and *Boerhaavia diffusa* (Jenifer *et al.*, 2012). Many compounds are known to accumulate in *in vitro* plant cell culture systems, and their concentrations are equal to or higher than that of the *in vivo* systems (Imaneh *et al.*, 2011). However, the development of targeted compounds by cell suspension culture can be very difficult due to high cell water content, persistent foaming in the bioreactor and poor metabolite processing (Baque *et al.*, 2011).

Studies by Ahmad *et al.* (2008) and Abdullah *et al.* (1998) showed continuous foaming in *Morinda citrifolia* cell suspension cultures using large-scale bioreactor, and this resulted in low yield of anthraquinones. The problem was partially avoided by replacing the explants with root cultures (Baque *et al.,* 2013). In some cases, the metabolites were absence in the cell biomass of root culture, or if present is secreted into the spent liquid media as reported by Jalil *et al.* (2015). Nevertheless, adventitious roots culture could serve as natural "bioactive substance factories" that grow vigorously in formulated medium, and possesses great potential to produce plant derived compounds.

The cultivation of adventitious root is a complex process influenced by many genetic and enviromental factors (Sorin *et al.,* 2005). Bienaime *et al.* (2015) stated that plant growth regulator (PGR) are essential in biomass growth and secondary metabolites

production. There are many examples of medicinally important species that using plant growth hormones to induce the adventitious roots for efficient development of secondary metabolites such as *Morinda citrifolia* (Baque *et al.,* 2013) and *Artemisia vulgaris* (Sujatha & Kumari, 2012). Normally, high auxin to cytokinin ratio favours root formation whereas high cytokinin to auxin ratio favours shoot formation (George *et al.,* 2008). However, applying combination of PGR with synergistic effects on the production of root biomass and secondary metabolites is scarcely explored.

So far, *in vitro* production of α-humulene, and simultaneous activity of elicitors on the development of secondary metabolites in root cultures of *Z. zerumbet* is yet to be reported. Thus, the aim of this study is to enhance growth of adventitious root cultures of *Z. zerumbet* Smith, and its production of α-humulene and zerumbone through synergistic effects of plant growth regulators and elicitors.

1.2 OBJECTIVES

a) To enhance the growth of adventitious root cultures of *Zingiber zerumbet* Smith through combined effects of plant growth regulators;

b) To optimize the production of α-humulene and zerumbone in adventitious root cultures of *Z. zerumbet* Smith by synergistic application of elicitors;

c) To improve adventitious root cultures, α-humulene and zerumbone production using balloon-type bubble column bioreactor (BTBCB).

CHAPTER 2: LITERATURE REVIEW

2.1 Plant description

2.1.1 *Zingiber zerumbet* Smith

Genus Zingiber is a large herbaceous genus comprised of 141 species that belongs to the family Zingiberaceae. *Zingiber zerumbet* Smith is known to be among popular species in this genus and usually used as traditional remedies and food additive either as a spice or flavour in Asia, India, China, and Saudi Arabia. *Z. zerumbet* is a tuberous herb plant that scattered naturally in damp or hill slopes like thickets. It is called by many name depends on the region, for instance, "Lempoyang" (Malaysia and Indonesia), "Awapuhi" (Hawai),"Ghatian and Yaimu" (India) and also known as shampoo ginger due to the presence of mucilaginous substance in the infloresence used by the hawaiians as natural hair shampoo and conditioner.

Approximately 1-2 meter tall, this plant can be categorized by the presence of pulvinus between the base of the petiole and ligule. The leaves are thin with 25-35 cm long and leaflets are arranged alternately along an arching pseudostem as shown in Figure 2.1. The most crucial part of this plant is the rhizome that is perennial, aromatic, thick and scaly which had been used traditionally as herbal medicine, food flavoring and appetizer. This plant has been established and widely distributed mainly in Asia and sub-tropical region (Yob *et. al.*, 2011).

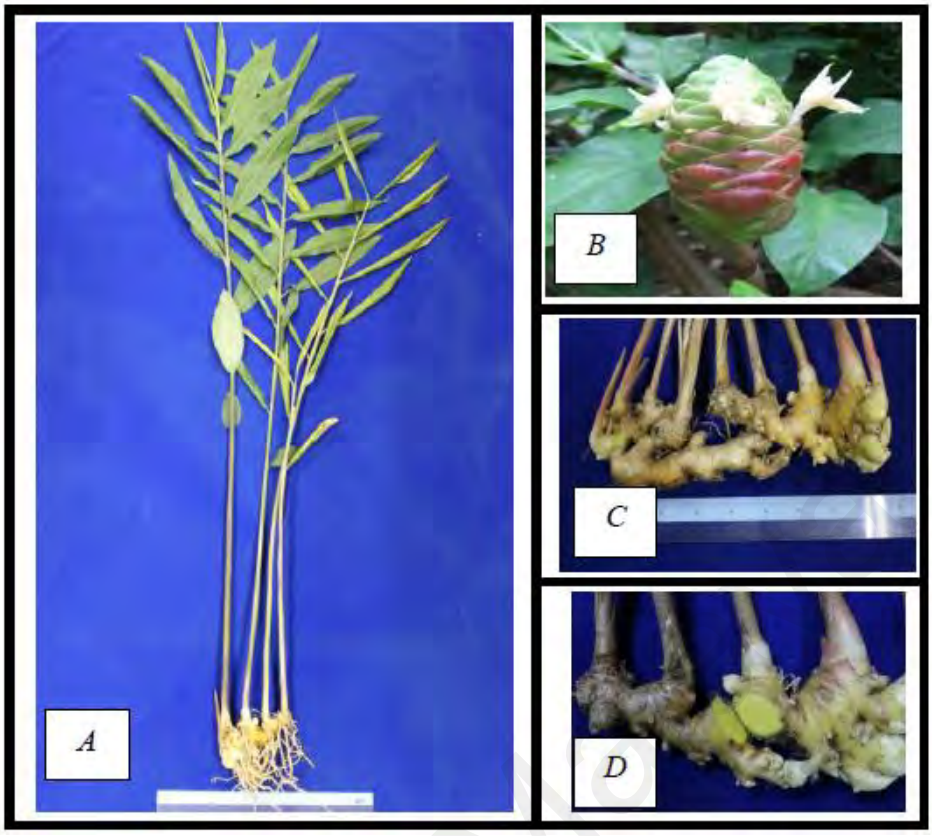

Figure 2.1: *Zingiber zerumbet* plants, *A*: whole plant of *Z. zerumbet*, *B*: inflorescence, *C*: rhizomes, *D*: pale yellow rhizome discoloration. (Jalil, 2018)

2.1.2 The importance of *Z. zerumbet* Smith

The rhizome of *Z. zerumbet* had become the center of investigation of all parts due to its medicinal properties. Since ancient times, the rhizome had been used to reduce inflammation, headache, toothache, stomach pain, digestive problems, diarrhea, and asthma (Yob *et. al.*, 2011). Over the years, scientists have revealed many pharmacological potentials of the rhizome such as anti-inflammatory (Akhtar *et al.,* 2019), anticancer, antibacterial, antipyretic (Girisa *et al.,* 2019), antioxidant and antimicrobial (Othman *et. al.*, 2019).

Interestingly, the ethanol extract of the rhizome play important role as immune suppressive agent that could significantly inhibit the migration of neutrophils, phagocytic

activity and production of reactive oxygen species in Winstar rats (Ghazalee *et al.,* 2019). Since 1944, there are many reports on the phytochemical content of *Z. zerumbet* rhizome started with the identification of humulene (Varier, 1944, Dash *et al.*, 2020), monoterpenes (Balakrishnan *et. al.*, 1956, Dash *et al.,* 2020) and zerumbone (Dev, 1960, Utaka *et al.,* 2020). Girisa *et al.* (2019) also stated that many phytochemicals possess valuable medicinal properties towards human diseases can be found from natural resources. Such compounds can be retrieved from conventional method and plant tissue culture method.

2.1.3 Tissue culture of *Zingiber zerumbet*

Plant tissue culture in Zingiberaceae *spp.* commenced in 1970 and later been done for micropropagation especially for commercial and endangered species such as *Alpinia galangal* (Borthakur *et. al.,*1998), *Etlingera elatior* (Faridah *et. al.,* 2011), *Kaempferia galangal* (Umar *et al.*, 2011), *Alpinia officinarum* (Kayalvizhi *et. al.,*2013), *Curcuma aeruginosa* (Theanphong *et. al*., 2010), *Boesenbergia rotunda* (Yusuf *et. al.,* 2011), *Zingiber montanum* (Hamirah *et. al*., 2010), *Costus speciosus* (Punyarani & Sharma, 2010), *Curcuma zedoaria* and *Zingiber zerumbet* (Stanly & Kang, 2007, Jalil *et. al.,* 2015). Study had shown that plant cell technology can be a powerful tool in producing the potential bioactive compound such as Zingiberaceae cells suspension (Yusuf *et al.*, 2013; Jalil *et al.*, 2015) and roots culture (Raju *et al.,* 2015). Recently, chemical constituents from the flowers, leaves and stems of *Zingiber striolatum* Diels had been extracted and their cytotoxic activities had been discovered (Tian *et al.*, 2020).

In vitro propagation and regeneration of embryogenic cell suspension of *Z. zerumbet* started with the study done by (Idris *et al*. 2009) and Faridah *et al.* (2011) where the

rhizome buds were used as explants. Further study were done by Jalil *et al.,* 2015 on the suspension culture and adventitious root culture (Jalil, 2018) of *Z. zerumbet* that can be exploited to produce the important compound known as zerumbone.

2.2 Plant tissue culture

Tissue culture is an *in vitro* method used to proliferate cells. Tissue cultures involve plant and animal cells and produce clones in which all genotypic cells produce the same genotype as the explants used. However, genotypic changes can be observed if mutations occur throughout the cultivation process. Plant Tissue Culture started since 1756 where the formation of callus on wounded plant discovered by Henri-Louis Duhumel du Monceau. A German-scientist, Gottlieb Heberlandt later found totipotency in plant during his work in isolating the mesophyll cells of Lamium. Plant Tissue Culture can be defined as a collection of method to grow plant tissues, cells and organs under aseptic condition on a media of known composition. The commercial use of tissue culture cloning was done on orchids in the 1920s. Subsequently, the tissue culture was rapidly expanding in the field of research after the introduction of the MS (Murashige & Skoog) media in 1962.

There are many advantages of using tissue culture such as multiplying and cloning from single tree to thousands of trees in a single year. It can be seen that this technique is capable of generating new cultivars widely and rapidly without affecting the existing plant. Therefore, plants threatened with extinction can be safely cloned. This plant tissue culture technique can also produce virus-free plants or other plant diseases because the explants used are of new meristem tissue that had been sterilized. Later to this day, plant tissue culture had been used for rapid mass propagation (Dash *et al.*, 2020) conservation of endangered plant species , production of disease-free plants (Popova *et. al.,* 2020) and

duplication of exact plant species (Arigundam *et al.,* 2020) and compound extraction (Tian *et al.*, 2020). There are many types of tissue culture that have been discovered and commonly used. Among the types are meristematic culture, cell suspension culture, protoplast culture and adventitious root culture.

2.2.1 Meristem culture

In the culture of the meristem, the apex meristem of the shoot can be cultured to produce disease-free explants. This is because on the meristem side were sterilized. However, techniques using meristem cultures are better suited for herb plants than for woody plants. Generally, the buds contain an active meristem located on the leaf surface that is more capable of growing into shoots. This technique can easily applied for mass propagation and the most recent work were done on five different strawberry cultivars (Naing *et al.*, 2019).

There are two types of meristematic bud cultures that can be used in propagation which are stem or single node and axilliary bud culture. For stem bud culture, the bud that attached to the stem were sterilized and cultured. These buds are cultured on nutrient media with appropriate antibiotics supplemented with low dose of cytokinins to produce shoots. Meanwhile, in axillary bud culture method, buds found at the axil of the leaf were sterilized. The buds were inoculated in media with high concentration of cytokinin to stops the apical dominance resulting in shoot growth. However, this is influenced by the types of the plant and the stage of the explants used. Generally, young explants need less cytokinin as compared to mature explants in shoots propagation. Shoot regeneration from axillary buds of field-grown papaya (*Carica papaya* L.) were done on MS medium

containing 2.0 mgL^{-1} BAP and 0.1 mgL^{-1} NAA later produced highest number of shoots (70.0) per explant (Veena *et al.*, 2015).

2.2.2 Protoplast culture

Protoplasts could be obtained by enzymatic digestion from plant tissues or cultured cells to eliminate the cell walls. The effectiveness of protoplast isolation depends in particular on the tissue conditions and the mixture of the enzymes to be used. Usually two or more protoplasts were combined to form a complete new organism in which it is able to survive and grow into mature new plants. The main purpose of protoplasmic culture is to produce hybrid plants. The advantage of using this culture technique is the production of plants with the desired characteristics of different plant species and to enable plants to become sexually hybrid. There are many studies on protoplast culture such as protoplast isolation for gene expression and protein interaction in pineapple (*Ananas comosus* L.) experiments recently done by Priyadarshini *et al.* (2018).

2.2.3 Cell suspension culture

When organogenesis happens by the development of a callus or a suspension in cell culture, it is called indirect organogenesis. Callus is a cell that has not been organized into a more specific cell. Callus development can be formed for subsequent organogenesis from several explants (leaves, roots, cotyledons, stems, floral petals). Due to the nature of the callus, the growth usually depends on several factors including the type of explants, the type of hormone applied, the concentration of the hormone and the environmental conditions such as humidity, temperature and etc. Callus culture are often used in

producing embryogenic cultures in plants such as *Phyllostachys heterocycla* var. pubescens (Moso bamboo) done by Yuan *et al.,* 2013.

Embryonic culture is a technique that uses two types of embryos: mature embryos and immature embryos. In some plant seeds, the maturation of the seeds may be due to chemical inhibitors or even to the structure of the embryo itself, for example the layers that protect the embryo. This problem can be avoided by using embryos as an explant and placed on nutrient media containing growth hormones to help the embryo grow into seed. This type of culture had been manipulated over the years for various species due to several biological and scientific advantages i.e. the development of commercially valuable plants, as well as the study of physiological and biochemical changes linked to the nature of a plant. To date, studies had been done to explore the process of somatic embryogenic induction in plant species such as carrot, rice (Garcia *et al.*, 2019) alfalfa (Sangra *et al.,* 2019) and corn (Salvo *et al.*, 2014).

Cell suspension cultures is a type of culture in which single cells or small aggregates of cells enlarge by transferring the callus / tissue to the liquid media and placing it on a 'gravitory shaker'. This is to allow for ventilation and diffusion of cells thus multiplying the cell. To obtain a perfect cell suspension, a friable callus is most typically inoculated into an agitated liquid medium in which it breaks up and disperses. Only single and small cells were maintained after eliminating the large callus and transferred to fresh medium. The suspension can then be propagated from an aliquot to fresh medium by regular subculture. This method will provide several valuable knowledge on cell biology, biochemistry, biochemical activities at the individual cell level and small cell aggregates.

Suspension culture established from important medicinal plants can be analysed for the development of secondary metabolites such as alkaloids, and significant industrial effort were done in exploiting and expanding this field. Yue *et al.* (2014) had compiled the usage of medicinal plant cell suspension cultures, the pharmaceutical applications and high-yielding strategies for the desired secondary metabolites. However, adventitious root cultures are more stable in the production of highly active compounds with mass propagation as compared to cell culture (Sivakumar, 2005; Jalil, 2018).

2.2.4 Adventitious root culture (AdRC)

There are two types of root culture that are commonly used in plant tissue culture which are hairy root culture and adventitious root cultures (AdRC). Hairy roots are developed from original roots while adventitious roots are those that developed from other plant organs. Adventitious roots do not require genetic modification compared to hairy root cultures and extraction is relatively reliable because no opine-like substrates are generated. Adventitious root cultivation techniques can also be conveniently utilized instead of hairy root methods since they are easier and healthier (Gaosheng and Jingming, 2012). AdRC can be generated via direct pathway (from cambium cell) or indirectly (from callus tissue).

AdRC are the best plant parts that suited for as natural "bioactive substance factories" and conversation of plant genetic resources since it can grow vigorously in phytohormone-free media, ease of excision and the ability to undergo morphogenesis (Popova *et al.,* 2020). Besides, Sivakumar *et al.* (2005), reported that adventitious root cultures can give a stable secondary metabolite production and grow well in physicochemical conditions as compared to cell suspension culture. Many researches had

been done on in-vitro adventitious root as it can serve as an alternative source to various phytochemicals that contribute to pharmaceutical, cosmetic and natural health product industries. For instances, the production of anthraquinones and phenolic compounds in adventitious root culture of *Morinda coreia* (Kannan *et al.*, 2020), accretion of phenolics and flavonoids content in AdRC of *Eurycoma longifolia* (Cui *et al.,* 2020), hypericin biosynthesis in AdRC of *Hypericum perforatum* (Tavakoli *et al.*, 2020), glycosides production in AdRC of *Stevia rebaudiana* (Ahmad *et al.,* 2020) and ginsenosides synthesis in AdRC of *Panax ginseng* (Hao *et al.,* 2020). Adventitious roots are induced by plant growth regulators (PGRs) that responded to mechanical damage in explant during tissue culture process.

Studies on plant growth regulators (PGRs) as an inducer for adventitious root cultures in many species has been reported including *Rumex crispus* (Mahdieh *et al.*, 2015), *Plumbago rosea* (Silja & Satheeshkumar, 2015), *Passiflora pohlii* (Simão *et al.*, 2016), *Couroupita guianensis* (Manokari & Shekhawat, 2016), *Ophiorrhiza mungos* L. (Deepthi & Satheeshkumar, 2017), *Stevia rebaudiana* (Radic *et al.*, 2016, Ahmad *et al.*, 2020) and *Morinda coreia* (Kannan *et al.*, 2020). Recently, shoot buds micropropagation of *Zingiber zerumbet* had been investigated by Gandhi & Saravanan (2019) meanwhile metabolite profiling, antioxidant activities and chromatographic determination of bioactive molecules in *in-vitro* cultures had been reported by Chavan *et al.* (2018).

2.3 Plant growth regulators (PGRs)

Plant growth regulators (PGRs) can be defined as any substance or mixtures of substances that produced naturally by plants used to regulating their own growth. They can be referred as phytohormones or plant hormones (Patel *et al.,* 2018). Ethylene was

the first plant growth regulator to be found in United States and used to enhance flowering in pineapple during 1930 (Bartholomew, 2014). These PGRs can be categorized as plant growth promoters such as auxins, gibberellins, cytokinins that important in controlling or modifying plant growth processes, formation of leaves and flowers, elongation of stems, development and ripening of fruits and plant growth inhibitors such as ethylene and abscisic acid that responsible in dormancy, abscission and senescence in plants (Rademacher, 2015). *Arabidopsis thaliana* and *Gossypium hirsutum* were used by Zhou *et al.* (2016) in order to discover the signalling mechanisms between the PGR and the development of plant cells.

2.3.1 Gibberellins

Gibberellins were developed in 19th century during the studies on rice disease that cause excessive elongation of stems and leaves due to fungal infection. This disease then led to the secretion of fungus *Gibberella fujikuroi* as the gibberellins was derived as the active component. All known gibberellins are diterpenoid acids which are synthesized in plastids through the terpenoid pathway and modified in the endoplasmic reticulum and cytosol before they form their biologically active form. Gibberellins (GAs) regulate several different aspects of plant growth and growth throughout the plant's life cycle i.e fostering cell division and elongation, seed germination, stem and hypocotyl elongation, root growth and flowering induction (Sun, 2011, Vera-Sirera *et al.*, 2016).

2.3.2 Cytokinins

Gottlieb Haberlandt was first to discovered that a compound (cytokinin) found in the phloem was capable of inducing cell division in 1913 (Kieber, 2002). Cytokinins are

Compounds with an adenine-like structure that facilitate cell division and have similar functions to kinetin. Kinetin was the first cytokinin discovered which isolated from herring sperm (Miller *et al.*, 1955) and this compound was named kinetin because of its ability to promote cytokinesis.

In 1961 Miller isolated the first naturally occurring cytokinin from corn called zeatin (Amasino, 2005). Since then, many cytokinins were detected in almost all higher plants as well as in mosses, mushrooms, bacteria, and also in tRNA of many prokaryotes and eukaryotes. The concentrations of cytokinin are highest in meristematic regions and areas with continuous growth potential such as roots, young leaves, fruit production, and seeds (Osugi & Sakakibara, 2015; Zürcher & Müller, 2016). Cytokinin is synthesized through the biochemical modification of adenine in the root and translocated via xylem to shoot (Sasaki *et al.*, 2014).

6-Benzylaminopurine, benzyl adenine, BAP or BA is a first-generation synthetic cytokinin usually used as a supplement in plant growth media such as Murashige and Skoog medium. Despite activating cell divisions, cytokinin can stimulates morphogenesis in tissue culture (shoot initiation/bud formation), the growth of lateral buds-release of apical dominance, improves the opening of the stomata in certain species and facilitates the transfer of etioplasts to chloroplasts by stimulation of chlorophyll synthesis (Kieber & Schaller, 2014).

2.3.3 Ethylene

Since ancient times, farmers would lighting kerosene lamps or burn things to ripen their crops. Frank E. Denny discovered in 1924 that it was ethylene molecule released by

kerosene lamps that contributed to the ripening. However, Crocker suggested in 1935 that ethylene was the plant hormone responsible for both fruit maturation and vegetative tissue senescence (Chamovitz, 2020). Ethylene is known as multifunction plant hormone that controls both cell development and cell death depending on its concentration, the plant species and timing of application that later promote or inhibit growth and senescence process.

For instance, application of ethephon, a compound releasing ethylene facilitated the evolution of ethylene and enhanced region of the mustard leaf at a lower dose while inhibiting it at a higher dose (Khan *et al.*, 2008; Iqbal *et al.*, 2017). Ethylene also controls the growth of leaves, flowers and fruits that encourage, supress or cause cell death, depending on the optimal or semi-optimal amounts of ethylene (Pierik *et al.*, 2006; Iqbal *et al.*, 2017).

The role of ethylene in the transition from vegetative to reproductive growth in Arabidopsis was identified through the comparison of ethylene-related mutants with the wild-type (WT) (Ogawara *et al.*, 2003). Exogenous application of ethylene or its biosynthetic analogue can accelerates the senescence of flowers in China. On the other hand, ethylene biosynthesis inhibitors such as amino oxyacetic acid can delayed the petal fall of flowers during senescence (Trivellini *et al.*, 2011; Iqbal *et al.*, 2017).

2.3.4 Abscisic acid

Abscisic acid (ABA) is an isoprenoid phytohormone discovered at least 50 years ago that controls various biological processes ranging from stomatal regulation to protein concentration and provides adaptation to different environmental stress conditions

including drought, soil salinity, cold tolerance, freezing tolerance, heat stress and heavy metal ion tolerance (Ruth Finkelstein, 2013). ABA is produced via plastidal 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway formed by the mevalonic acid-derived precursor farnesyl diphosphate (FDP). After the cleavage of C40 carotenoids in MEP, the C_{15} backbone of ABA is produced.

The first precursor to ABA is zeaxanthin which resulted from the process of enzyme-catalyzed epoxidations and isomerizations. The second precursor, xanthoxin is produced by the dioxygenation reaction cleavage of the C_{40} carotenoid were further oxidized through abscisic aldehyde to ABA (Sah *et al.,* 2016). ABA signalling results in significant improvements in gene expression including changes in MrNA, transcription regulation and stabilization according to Cutler *et al.* (2010). ABA plays multiple roles in plants and also needed under stress-free conditions for plant growth and development. ABA supported root meristem maintenance as reported by Zhang *et al.* (2010) that ABA can promote the maintenance of stem cell by promoting of QC quiescence and the suppression of stem cell differentiation.

Several research have suggested that mutants with defects in ABA biosynthesis or signaling show altered or delayed senescence in Arabidopsis i.e. an identified a receptor kinase (RPK1) that mediates age-and ABA-induced senescence in old leaves (Lee *et al.*, 2011).

2.3.5 Auxins

Plant synthesizing hormone such as auxin usually found in shoot and root tips that responsible in promoting cell division, stem and root growth in response to gravity and

light. Many studies had been done extensively for this plant group hormone due to its biological functions (Zhao, 2010). In shoot elongation, auxin influence the existing gibberellins to promote the cell growth and increase the spacing between the nodes and branch.

For seed germination, the shoot grows depends on the abundance of auxin in that area of cell and their interactions within the plants whether to grow into the soil or laterally, towards the light. Auxins also can be applied to cut stem in order to initiate roots. Meanwhile, auxin in the flower helps to promote the development of the fruit by causing maturity of the ovary wall. In addition, the efficacy of auxins may be affected by cuttings physiological status as regards carbohydrates and/or other nutrients (Costa *et al.,* 2017).

Charles Darwin and his son, Francis were the first to discover auxin by performing experiments in coleoptiles that exposed the sheaths to the light in 1881 (Hohm *et al.*, 2013). This experiments contributed to the discovery of indole-3-acetic acid (IAA) as the major naturally occurring auxin in plants. Several compounds with notable auxin activity were synthesized i.e. 2, 4-Dichlorophenoxyacetic acid (2,4-D), Naphthaleneacetic acid (NAA), Indole-3-butyric acid (IBA), Dicamba and Picloram (Enders *et al.,* 2015)

The most popular synthetic auxins are NAA and IBA that usually used to induce root growth. The root formation, however, mainly results from a balance between auxins and other hormones such as cytokinins and gibberellins. This balance not only influences root initiation but also root elongation, axillary shoot growth, and ultimately, the future shoot-to-root ratio of the new rooted plant and secondary metabolites secretion. Lately, there are many studies on various medicinal plant had been done where adventitious roots have been developed by optimizing the plant growth regulators as shown in Table 2.1.

Table 2.1: List of medicinal plant with targeted secondary metabolites wherein adventitious roots have been developed by using PGRs.

No.	Plant Species	Secondary Metabolites	Optimized Conditions (Media & PGRs)	References
1	*Aloe vera*	Aloe-emodin & Chrysophanol	B5 +0.5 mgL^{-1} NAA+ 0.2 mgL^{-1} BA	Lee *et al.* 2011; 2013
2	*Boesenbergia rotunda*	Pinostrobin	MS+0.5 mgL^{-1} NAA	Azhar *et al.* 2018; Yusuf *et al.* 2018
3	*Camellia sinensis*	Catechin & Caffeine	½ MS	Kim *et al.* 2013
4	*Chlorophytum borivilianum*	Stigmasterol & Hecogenin	MS+3 mgL^{-1} IBA	Bathoju & Giri 2012
5	*Costus igneus*	Resinoid	MS+0.5 mgL^{-1} IBA	Nagarajan *et al.* 2011
6	*Couroupita guianensis*	Eugenol & Farnesol	½ MS+2 mgL^{-1} IBA	Manokari & Shekhawat 2016
7	*Fagonia indica*	Apigenin, Gallic acid, Rutin	MS+1 mgL^{-1} NAA	Khan *et al.* 2017
8	*Gynura procumbens*	Kaempfrerol & Myricetin	MS+3 mgL^{-1} NAA+1 mgL^{-1} IBA	Faizah *et al.* 2018
9	*Hypericum perforatum*	Hypericin, Quercetin, Hyperoside	MS+1 mgL^{-1} IBA	Cui *et al.* 2010
10	*Labisia pumila*	Flavonoids & Phenolics	MS+5 mgL^{-1} IBA	Hasan *et al.* 2014
11	*Prunella vulgaris*	Prunellin	MS+0.5 mgL^{-1} NAA	Fazal *et al.* 2014
12	*Luffa acutangula*	Luffin	MS+1 mgL^{-1} IBA+1 mgL^{-1} NAA	Umamaheswari *et al.* 2014
13	*Perovskia abrotanoides*	Tanshinone	MS+2 mgL^{-1} NAA	Zaker *et al.* 2015
14	*Podophyllum hexandrum*	Podophyllotoxin	MS+1.5 mgL^{-1} IBA	Rajesh *et al.* 2012
15	*Polygonum multiforum*	Antrhaquinones, Phenolic, Flavonoid	MS+9.4 µM IBA	Ho *et al.* 2018
16	*Panax quinquefolium*	Ginsenoside	MS+3 mgL^{-1} IBA+1 mgL^{-1} NAA	Wang *et al.* 2016
17	*Psammosilene tunicoides*	Triterpenoid Saponin	B5+0.05 mgL^{-1} IBA+0.1 mgL^{-1} NAA	Zhang *et al.* 2017
18	*Rumex crispus*	Antrhaquinones, Flavonoids	MS+5 µM NAA	Mahdieh *et al.* 2015
19	*Talinum paniculatum*	Saponin	MS+2 mgL^{-1} IBA	Solim *et al.* 2017
20	*Withania somnifora*	Whitanolide	½ MS+ 0.5 mgL^{-1} IBA+0.25 mgL^{-1} IAA	Thilip *et al.* 2015

2.4 Plant secondary metabolites

Natural products are substances derived from various organisms and microorganisms including plants, animals, bacteria and fungi. Such products that produced were identified as primary metabolites and secondary metabolites. Primary metabolites are the essential

compounds that used for growth such as carbohydrate, lipids, amino acids, enzymes, nucleic acid, ascorbic acid and protein while secondary metabolites are the compounds produced due to some enzymatic process of primary metabolites and some of them derived from the defences mechanism such as of alkaloids, phenolics, sterol, steroids, essential oil, tannin, terpenoid, flavonoids and many others (Hussein and El-Anssary, 2019). There have been huge interest in the use of plants in medication and plant-based drugs since the awareness of the health hazards and toxicity of the synthetic drugs (Ahmad *et al.*, 2015).

These secondary metabolites production could give a great contribution to the economic importance of plants due to its phytomedicine potential even though the accumulation usually lower than the primary compounds. Secondary metabolites are of low molecular weight and not essential for normal plant growth and development but it is served as a protector for the plant to survive in its environment (Tiwari & Rana, 2015). There are four main classes that can be classified for this plant metabolites which are phenolic compounds, terpenoids, alkaloids and sulphur-containing compounds.

2.4.1 Terpenes

Terpenes is the main group of this metabolites which consist of five units of carbon isoprene and are attached in thousands of ways with several isoprene units. Terpenes are basic hydrocarbons, whereas terpenoids are the altered terpenes that has distinct methyl groups and functional groups that shift or detach at different positions. There are three main categories for terpenoids depending on its carbon units which are monoterpenes, sesquiterpenes, diterpenes and triterpenes as shown in Figure 2.2. These compounds and

their derivatives are used as medicinal products i.e. artemisinin from *Artemisia annua* that used to treat malaria and other associated compounds (Badshah *et al.,* 2018).

A lot of flavourings and good fragrances are made of terpenes because of the pleasant scent. On the other hand, terpenoids with their structural variations are biologically active and are used widely to treat many illnesses and play a major role in food, drugs, cosmetics, hormones and vitamins. Besides, some terpenoids have suppressed certain human cancer cells and are used as prescription drugs in cancer treatments like taxol and its derivatives from *Taxus baccata* (Malik *et al.*, 2011).

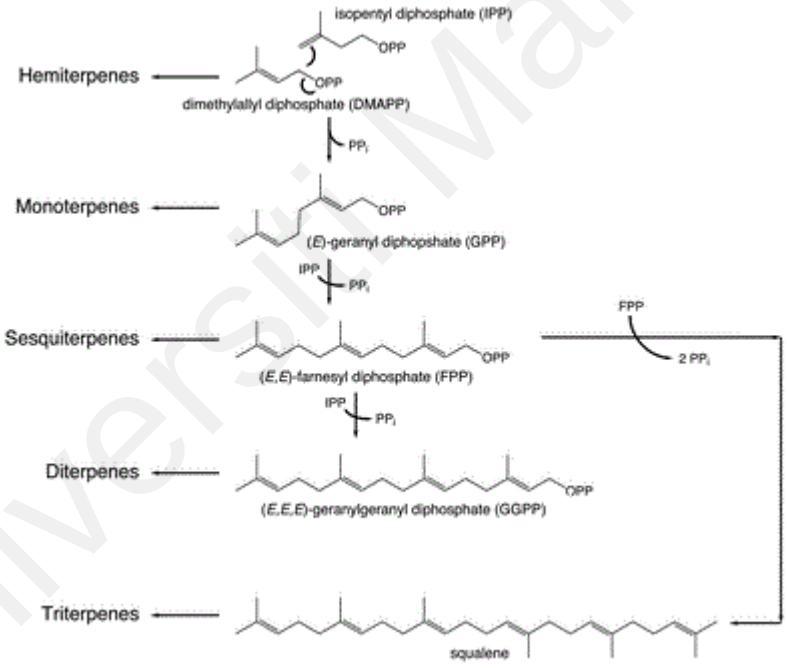

Figure 2.2: Classification of terpenes. Source: Gao *et al.* (2012)

Monoterpenes constructed from 10 carbon atoms conjugated with two isoprene units and their molecular formula is $C_{10}H_{16}$. This compound can be categorized into monocyclic, acyclic and bicyclic based on its structure and can be extract from essential and fixed oils. These compounds exhibit strong smell and taste that had been used by many cosmetic companies. In fact, mixture of various monoterpenes that found in oils

were used as fragrances in making perfumes. Monoterpenes are the major components in the essential oil of Perilla frutescens var. crispa with diverse biological effects including antifungal, neuroprotective, anticancer, angiogenesis inhibitory, anti-inflammatory, and antioxidant activities (Nam *et. al.*, 2017).

Sesquiterpenes are the secondary metabolite class composed of three units of isoprene ($C_{15}H_{24}$) and can be found in cyclic, bicyclic and tricyclic and also linear forms. Many of the latex containing sesquiterpene in plants are potent antimicrobial and anti-insecticide agents (Chadwick *et al.,* 2013). *Artemisia annua* shoots and roots possess a sesquiterpene known as Artemisinin that has a great potential in treating malaria and other chronic diseases (Weathers *et. al.,* 2011). Cyclic sesquiterpene like zerumbone exerts antimitotic activity in human cancer cell, Hela cells line (Ashraf *et al.,* 2019).

Belong to a diverse group of chemical components, diterpenoids presents in different natural sources and possess four isoprene units with molecular formula of $C_{20}H_{32}$. Compounds of this class mostly used as anti-inflammatory, antimicrobial, anticancer, and antifungal and cardiovascular activity, such as eleganolone, grayanotoxin, marrubenol, forskolin, and 14-deoxyandrographolide (Zhang *et al.,* 2017).

Triterpenes contains of 30 carbon atoms consist of 6 isoprene units and considered as a major class of secondary metabolites. It has several methyl groups which can be oxidised to carboxylic acids aldehydes and alcohols that make it more stable and biologically distinct. Xuedanencins is a triterpenes that can be found in Tubers of *Hemsleya penxianensis* possess cytotoxic activity against the Hela human cancer cell line (Li *et. al*., 2017).

2.4.2 Phenols

Being the main class of secondary metabolites in plants, this organic compounds are distinguished by a carbon atom with a hydroxyl (—OH) group as a part of its ring. Phenol also known as monohydroxybenzene (C_6H_5OH) as well as benzenol, or carbolic acid. It can be divided into phenolic acids and polyphenols when combined with mono- and polysaccharides, linked to one or more phenolic group, or can occur as derivatives, such as ester or methyl esters.

Most phenols present in nature such as tyrosine as amino acid found in proteins (Parthasarathy *et al.*, 2018), epinephrine as stimulant hormone produced in adrenal medulla) and urushiol as compounds secreted by poison ivy to prevent animals from eating the leaves(Lott *et al.*, 2019). Some of complex phenols that found in essential oils of plants are being used in food flavourings i.e vanillin that isolated from vanilla beans (Zhang *et al.,* 2014) and salicylate (minty taste) isolated from wintergreen (Michel *et al.,* 2019). Among the different groups of phenolic compounds, the primary dietary phenolic compounds are phenolic acids, flavonoids and tannins.

Many studies have shown a strong and significant connection between the phenolic compound content and the fruit and vegetable antioxidant potential (Reddy *et al.*, 2010). The antioxidant feature, found in plants, plays an important role in reducing lipid degradation in (plant and animal) tissues as it not only maintains the consistency of the food taste when integrated in the human diet, it also decreases the risk of developing many diseases. Research have shown that a diet high in fruit and vegetables helps to slow the ageing cycle and that the risk of inflammation and oxidative stress associated with chronic

diseases i.e. coronary conditions, arteriosclerosis, obesity, diabetes, cataracts, cognitive control disorders, and neurological diseases (Tanaka *et al.*, 2012; Pojer *et al.*, 2013).

2.4.3 Alkaloids

Alkaloid is a nitrogen-containing organic bases family that exists naturally. Alkaloids have significant and complex biochemical effects on living organisms such as nicotine, morphine, strychnine, ephedrine, and quinine. Alkaloids are mainly present in plants, and abundant in some flowering plant families. In general, there are only a few classes of alkaloids in a given genus, but both ergot fungus (*Claviceps sp*.) and opium poppy (*Papaver somniferum*) that produce more than 50 different varieties. The Amaryllidaceae (amaryllis), Ranunculaceae (buttercups) and Solanaceae (nightshades) are other popular families that contain alkaloids.

The alkaloids roles in the lifecycle of plants were recently discovered and research indicates that they can represent different biological functions. In certain plants, alkaloid concentration rises just before seed forming and then falls off when the seed is mature, showing that alkaloids may play a role in this process (Schramm *et al.*, 2019). Many alkaloids in their pure form are colourless, non-volatile, crystalline solids and appear to have a bitter flavour. Morphine is the potent active constituent of the *Principium somniferum* and was the first alkaloid to be extracted (Krishnamurti & Rao, 2016).

Alkaloids possess many medicinal properties such as morphine which is powerful drug used for the relief of pain despite of the addictive effects, Quinidine obtained from plants of the genus Cinchona were used to treat arrhythmias, ergonovine (ergot alkaloids) is used to reduce uterine hemorrhage after childbirth and ephedrine is used to relieve the

discomfort of common colds, sinusitis, hay fever, and bronchial asthma (Dash *et al.,* 2020). Many alkaloids have local anaesthetic properties although they are rarely used for this purpose clinically. Cocaine (from *Erythroxylon coca*) is a very potent local anesthetic (Tsuchiya, 2017), quinine (from Cinchona) is a powerful antimalarial agent that was formerly the drug of choice for treating that disease (Achan *et al.,* 2011), curare (from *Chondrodendron tomentosum*) is used as a muscle relaxant in surgery (Raghavendra, 2002) meanwhile vincristine and vinblastine (from *Vinca rosea*) are widely used as chemotherapeutic agents in the treatment of many types of cancer (Lu *et al.*, 2012).

However, some alkaloids are illicit drugs and poisons i.e. nicotine obtained from the tobacco plant (*Nicotiana tabacum*) is the main addictive ingredient of the tobacco smoked in cigarettes, cigars, and pipes, hallucinogenic drugs mescaline (from *Anhalonium spp*.) and psilocybin (from *Psilocybe mexicana*) and synthetic derivatives of the alkaloids morphine and lysergic acid (from *Claviceps purpurea*) produce heroin.

2.4.4 Sulphur containing compound

Organosulfur compounds are chemical compounds comprising sulphur. They are also correlated with foul-smelling contaminants, and many of the sweet substances are identified as organo-sulphur derivatives. Nevertheless, not all organo-sulphur is life-threatening. Gliotoxin is a sulphur-containing mycotoxin produced by several species of fungi under investigation as an antiviral agent (Smith & Calvo, 2014). Compounds such as allicin and ajoene are responsible for the scent of garlic, and lenthionine corresponds to the flavour of shiitake mushrooms.

Many of these natural products also have important medicinal properties such as preventing platelet aggregation or fighting cancer. Besides, fossil fuels, iron, gasoline, and natural gas from ancient organisms naturally produce compounds of organosulphur, the degradation of which is a major focus of oil refineries. In fact, the specific antibiotics, penicillin and sulfa, also comprised of sulphur. Although several lives are protected by sulphur-containing antibiotics, sulphur mustard can be a lethal chemical warfare weapon (Etemad *et al.,* 2019).

2.5 Secondary metabolites in *Zingiber zerumbet*

Many high-value secondary metabolites with various applications are extracted from plants. Some compounds can be obtained from naturally produced plants, but often there are geographical and environmental constraints that may hinder industrial development and the conventional cultivation of certain plant species is difficult or may take many years. Besides, based on the research that had been conducted by Ramakrishna and Ravishankar (2011), environmental conditions other than infestation, diseases and application of pesticides affects the quality and quantity of the secondary metabolite collected from wild and field grown plants. Thus, plant cell and tissue culture methods tend to be environmentally sustainable approaches to the development of secondary metabolites where natural sources are minimal or where chemical synthesis is not feasible.

Plant tissue culture techniques offers mass propagation of plants in aseptic and environmental regulated environments and the large-scale development of secondary metabolites throughout the year without seasonal restrictions. Besides, the cultivation of *in vitro* adventitious roots had been introduced as an alternative for natural compounds

production (Zhang *et. al.,* 2012). Adventitious roots cultures is the perfect tools to be adopt in producing large quantity of natural bioactive compounds because of its characteristic that are easy-to-grow in phytohormone-free media and possess great potential to produce plant derived compounds. By these techniques, secondary metabolites are isolated and produced in large quantities, as well as of good quality and are stable and produced rapidly in an adapted culture medium (Ahmad *et al.,* 2015).

Moreover, many compounds are known to accumulate in *in vitro* plant cell culture systems, and their concentrations were equal to or higher than that of the *in vivo* plant (Imaneh *et. al*., 2011). Adventitious roots have been successfully induced in many plant species and cultured for the production of high value secondary metabolites of pharmaceutical, nutraceutical and industrial importance such as *Panax ginseng*. Besides, Wilson *et al.* (2012), reported that plant cell culture can give a stable secondary metabolite production and grow well in physicochemical conditions as compared to conventional method.

To date, there are few valuable metabolites that had been produced on a large scale such as ginsenosides from *Panax ginseng* (Baque *et al.*, 2012). Eleuthrosides B & E, chlorogenic acid phenolic, flavonoid from *Eleutherococcus koreanum* (Lee *et al.*, 2015), sphaeralcic acid (anti-inflammatory compounds) from *Sphaeralcea angustifolia* (Pérez-Hernández *et al.*, 2019).

Secondary metabolite of *Z. zerumbet* had been reported to possess biological activities such as antimicrobial, anticancer, anti-inflammatory, antinociceptive, antiulcer, antoxidant, antihyperglycemic and antiallergic properties. From previous investigations, zerumbone has been found as one of the main compound in the rhizome *Z. zerumbet*

(Akhtar *et al.*, 2019). Zerumbone isolated from the rhizome can be used as an immunosuppressive agent by down regulated the engulfment of *Escherichia coli* (Jantan *et al.,* 2019) and promotes cytotoxicity in human tumor glioblastoma cells via reactive oxygen species generation (Jalili-Nik *et al.,* 2020). This compound also can act as antifungal agents by inhibits *Candida albicans* biofilm formation and hyphal growth (Shin *et al.,* 2019).

Zerumbone that predominant compound in this plant is believed to reduce HIV activity (Dai *et. al*., 1997), act as antiangiogenic agent in the treatment of hepatocellular carcinoma (Samad *et al.*, 2019) anti-inflamatory agent (Ghazalee *et. al*., 2019) and is also known to prevents cancer cell proliferation (Girisa *et. al*., 2019). Besides zerumbone, Tian *et al.* (2020) found other 36 compounds in the essential oil of dry *Z. zerumbet* rhizome such as *α*-humulene (29.4%), humulene oxide I (6.0%), humulene oxide II (3.9%), camphene (3.9%), *β*-caryophyllene (2.5%), camphor (2.4%), caryophyllene oxide (2.1%), and 1, 8-cineole (1.6%). As the second highest constituent in the rhizome, *α*-humulene could be the next interesting compound to discover. Recent study revealed that *α*-humulene extracted from plant can be used as antibacterial by inhibit the growth of *Bacteroides fragilis* cells and biofilm (Jang *et al.,* 2020).

2.5.1 Zerumbone

Zerumbone is a sesquiterpene exist in the form of rich milky exudates in the the rhizome. Zerumbone can be identified by three double bonds, two conjugated and one independent, and a double conjugated carbonyl group inside the 11-membrane ring structure or one ring with fifteen carbons and 220 hydrogen ($C_{15}H_{220}$). This chemical structure of zerumbone possess powerful latent reactivity that can generate a library of

six natural product-like skeletons by transannulation (Utaka *et al.*, 2020). Several IUPAC name were identified for zerumbone which are 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. Zerumbone can be categorized as polar and non-polar compound with molecular weight of 218.3 gmol^{-1}. Zerumbone can be melt at 65.3°C and can be isolated from fresh rhizomes by hydrodistillation (steam distillation) and recrystallization methods (Rahman *et al.*, 2014). Application of zerumbone is believed to treat chronic disease such as osteoarthritis, obesity, diabetes, diabetic nephropathy, diabetic retinopathy, chronic gastritis, neuropathic pain, atherosclerosis, inflammation and cancer (Singh *et al.*, 2019) and could be prophylactic alternative to prevent acute and chronic liver injury (Kim *et al.*, 2019).

Figure 2.3: Chemical structure of zerumbone. Source: Singh *et al.* (2019)

2.5.2 α-Humulene

Humulene which also known as α-humulene or α-caryophyllene is a naturally occurring monocyclic sesquiterpene ($C_{15}H_{24}$) which contains an 11-membered ring and consists of 3 isoprene units containing three non-conjugated C = C double bonds, two of

which are triple substituted and one of which is duplicated. The IUPAC name for this compound is 2,6,6,9-Tetramethyl^{-1},4-8-cycloundecatriene and it is also called as α-caryophyllene; 3,7,10-Humulatriene. Humulene is an isomer of β-caryophyllene, and it is usually present together as a mixture in many aromatic plants.

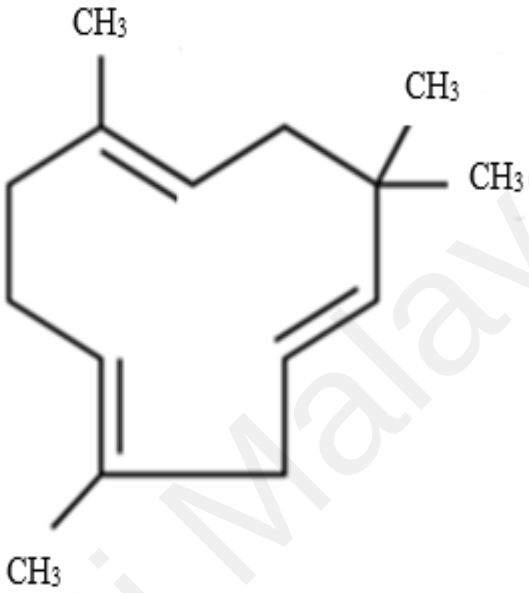

Figure 2.4: Chemical structure of α-humulene.

2.5.3 Biosynthesis pathway of zerumbone and α-humulene

The biosynthetic pathway of these compounds begins with the cyclization of (2*E*,6*E*)-farnesyl diphosphate. In the pathway, the terpene synthase catalyze the synthesis of α-humulene. Thereafter, the α-humulene is converted to 8-hydroxy-α-humulene by α-humulene^{-1}0-hydroxylase followed by conversion to zerumbone by zerumbone synthase. Since α-humulene is the intermediate to zerumbone, this study is focusing on presence of α-humulene and stress that related to the conversion of zerumbone. However, many improvement and studies has to be made to boost the production of these compounds by using elicitors and precursor as well as biosynthesis of the compound in order to attain commercial demand.

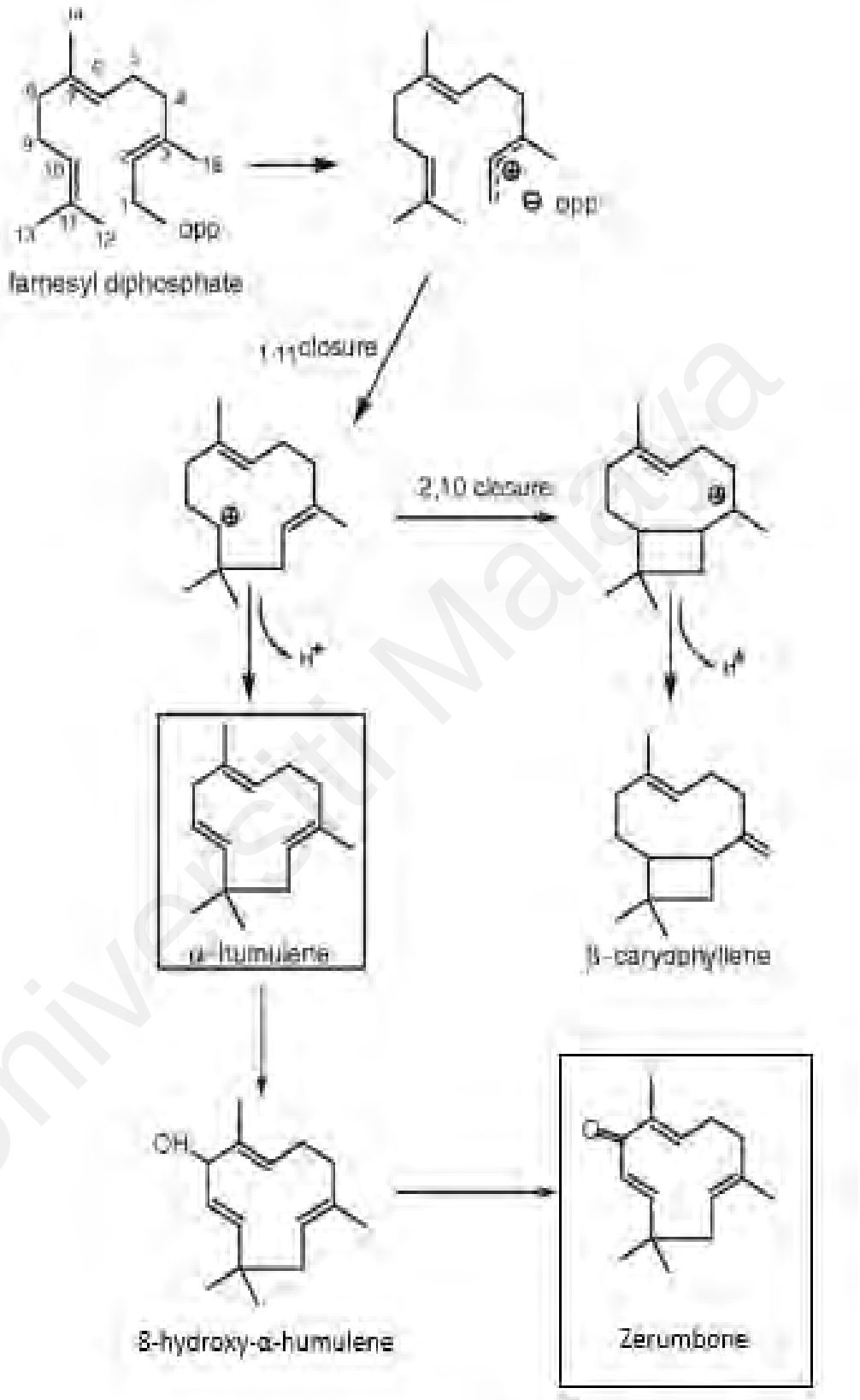

Figure 2.5: Biosynthesis pathway of zerumbone and α-humulene. Source: Yu *et al.* (2008)

2.6 Elicitation

Plants usually activate their defensive mechanisms via induction of secondary metabolites biosynthesis as hypersensitive responses and defensive barriers when react to pathogens, biotic and abiotic stresses (Vasconsuelo & Boland, 2007; Jeandet *et al.*, 2016). The use of elicitors stimulates plant defense mechanisms which have resulted in significant development of bioactive secondary metabolites (Jeandet *et al.*, 2016). For instance, four major boswellic acid isomers were found in *Boswellia serrata* callus cultures stimulated by the biotic and abiotic elicitors (Ghorpade *et al.*, 2011). Thus, elicitor can be defined as any chemicals that trigger mechanism in living organisms to produce secondary metabolites. Elicitors can be categorized into two types which are biotic elicitors including polysaccharides, bacteria, viruses, fungi and abiotic elicitors such as metal ions and inorganic compounds (chemical elicitors).

Generally, the exogenous supply of signal compounds (chemical elicitors) such as as jasmonic acid (JA), methyl jasmonate (MeJA), 2-hydroxyl ethyl jasmonate, salicylic acid (SA), acetyl salicylic acid (ASA), trifluro ethyl salicylic acid, ethylene (ET), nitric oxide (NO), sodium nitropruside (SNP), ethrel or ethephon (Ethe) could create a stress-like environment to a culture system of plants that trigger the secondary metabolites production and usually used for elicitation studies. Elicitors not only offer quantitative improvement (yield enhancement of existing compound) but also discovered to qualitatively synthesize new secondary metabolites by genetic and biochemical activities in the cellular background (Murthy *et al.* 2014; Ramirez-Estrada *et al.* 2016).

There are many factors that have to be taken care of to ensure the effectiveness of elicitor. The main factors are elicitor specificity, culture condition (growth stage, medium

composition and light), elicitor concentration and treatment interval. Besides, the physiological responses of adventitious root in relation to enzyme activity and stress level should also take into consideration More studies are then needed to understand this plant elicitor interaction and thereby the elicitation response.

Zhao *et al.* (2005) reported that this method has been the best approach in enhancing significant metabolite production (Jeandet *et al.*, 2016) of various plants so far. For instance, elicitation with salicylic acid to multiple shoot cultures of *Andrographis paniculata* increased accumulation of accumulated andrographolide content (Zaheer and Giri, 2015) and acetyl salicylic acid increased daidzin content by 2.3-fold (1.44 % DW) compared to untreated control (0.62 % DW) roots of *Psoralea corylifolia* when treated at 25 µM (Zaheer *et al.* 2016). MeJA elicitation at 100 µM increased taxane production in n hairy root cultures of Taxus x media var. Hicksii (Sykłowska-Baranek *et al.* 2015). 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 *Hypericum perforatum.*

Synergistic effect of elicitors on compounds accumulation was also reported. Previous results suggest that combination of elicitors and media enhancement treatment had a beneficial impact on the enhancement of secondary metabolite development in vitro (Giri and Zaheer, 2016). The mixture of MeJA and L-tyrosine increased the baine yield to 84.62 mg l^{-1} 6 days after treatment in *Papaver bracteatum* in cell suspension cultures (Zare *et al.* 2014) and combined treatment of JA and gibberellic acid promoted the development of improved secondary antioxidant metabolites in *Artemesia absinthium* L. cell suspension cultures (Ali *et al.* 2015). Among the combination treatment studies, methyl jasmonate (MeJA) and salicylic acid were frequently used. MeJA and SA promoted

sanguinarine production in *Argemone mexicana* cell cultures (Trujillo-Villanueva *et al.* 2012) and onfuranocoumarin production up to 4.7 and 5.9-fold shoot cultures of *Ruta graveolens* (Diwan and Malpathak, 2011). Recently, Largia *et al.* (2015) reported on the enhancement of bacoside A in shoot cultures of *Bacopa monnieri* through synegistic effect of MeJA and SA.

Addition of elicitors to plant cell cultures can impede biomass accumulation since elicitation shifts primary metabolism to secondary cell metabolism as seen in the root suspension culture of *Zingiber zerumbet* (Jalil, 2018) and *Talinum paniculatum* (Faizal and Sari, 2019). To overcome this issue the optimization on the growth medium, type of elicitor, supplementation time of elicitor need to be optimized and applied in the bioreactor system to attain commercial demand.

2.6.1 Methyl jasmonate

Methyl jasmonate (MeJA) is a volatile organic compound used for plant defence and a series of developmental processes such as seed germination, root production, flowering, fruit ripening and senescence. The IUPAC name for MeJA is cyclopentaneaceticacid, 3-oxo-2-(2-penten^{-1}-yl)-, methyl ester derived from jasmonic acid and the reaction is catalyzed by S-adenosyl-L-methionine: jasmonic acid carboxyl methyltransferase.

MeJA is an exclusive molecule with the proclivity to permeate interplant connection through airborne defence response signals (Dar *et al.* 2015). MeJA and its derivatives plays important roles as major signalling compounds in producing the secondary metabolites through elicitation process (Ramirez-Estrada *et al.*, 2016). However, MeJA is reported to interfere the transmembrane auxin flux pathway which cause inhibition to the root growth (Yan and Xie, 2015). Nevertheless, when MeJA were supplied at

optimized concentration, time and suitable incubation period with culture, minimal effect on the root growth and improvement on secondary metabolite could be achieved (Ramirez-Estrada *et al.*, 2016). Zhu *et al.* (2014) reported that adding MeJA increases celastrol content in hairy root cultures of *Tripterygium wilfordii*. Besides, elicitation with methyl jasmonate increases the accumulation of selected centellosides and phenolics in *Centella asiatica* (L.) Urban shoot culture (Skrzypczak-Pietraszek *et al.*, 2019).

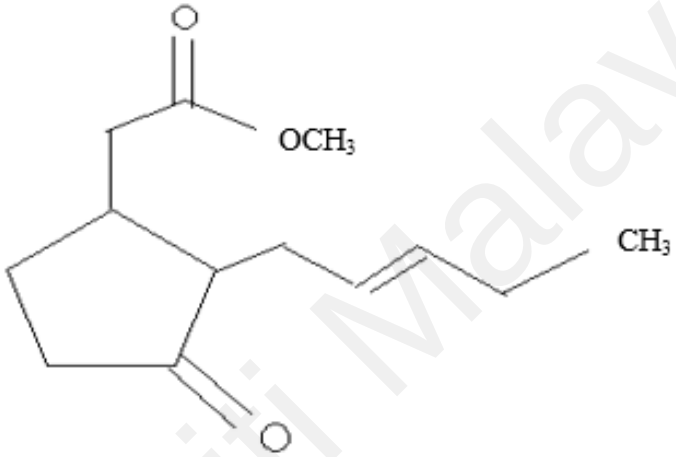

Figure 2.6: Chemical structure of methyl jasmonate.

2.6.2 Salicyclic acid

Salicyclic acid (SA) consist of carboxyl (−COOH) and hydroxyl (−OH) groups directly attached to an aromatic benzene ring, unlike a true β-hydroxy acid, which contains an aliphatic carbon atom chain. The IUPAC name for SA is 2-hydroxybenzoic acid or orthohydrobenzoic acid. It can be chemically synthesized or biologically found in most unicellular and multicellular organisms including plants such as willow bark, sweet birch, and wintergreen leaves (Arif, 2015). Several reports have recently given a comprehensive overview on SA biosynthesis (Dempsey *et al.*, 2011; Dempsey and Klessig, 2017; Klessig *et al.*, 2018). Genetic and biochemical data has demonstrated that SA can be derived from two different and compartmentalized pathways: the

isochorismate (IC) pathway discovered in the plastids and the phenylalanine ammonia-lyase (PAL) pathway in the cytosol that starts with the accumulation of chorismic acid resulted from shikimic acid biosynthesis (Maruri-López *et al.*, 2019).

In *in-vivo* plants, salicylic acid is a phenolic phytohormone and is found in plants with roles in plant growth and development, photosynthesis, transpiration, ion uptake and transport which involved endogenous signaling, mediating in plant defense against pathogens. Meanwhile, through *in-vitro* method, salicylic acid were used as an elicitor that is commonly known as a stressed molecular signal in plants to respond to the pathogens (Bernal-Vicente *et al.,* 2020). Scientist also noticed the potential of SA as plant growth regulator in the past two decades which contribute to the growth and development in plant including seed germination, vegetative growth, flowering, fruit yield, senescence, stomatal closure, thermogenesis, photosynthesis, respiration, changes in the alternative respiratory pathway, glycolysis and the Krebs cycle (Klessig *et al.,* 2018). This has been demonstrated in *Solanum tuberosum* L. which SA significantly increased the shoot number, leaf number, leaf area, root length and number as well as microtuber weight and number (Alutbi *et al.,* 2017).

The most recent research conducted by Pasternak *et al.* (2019) revealed that SA affects the biosynthesis of auxin and transport and alter the root meristem in *Arabidopsis thaliana* in which low-concentration SA facilitated adventitious roots whereas high-concentration SA cause inhibition in root growth. Besides, SA is also essential in the regulation of plant reactions to a number of abiotic stresses (climate change, salinity and drought). For example, SA mitigated the detrimental effects of salt stress in Ethiopian mustard, *Brassica carinata* (Husen *et al.,* 2018). SA act as signal molecule that activate genes and induces diseases resistance in most plants including tomato, *Lycopersicon esculentum*

(Jendoubi *et al.* (2017), pepper, *Capsicum annuum* (Chandrasekhar *et al.,*2017) and rice *Oryza sativa* (Stella de Freitas *et al.*, 2019). Besides, SA is also essential in the regulation of plant reactions to a number of abiotic stresses (climate change, salinity and drought). Salicylic acid were usually exploited to produce secondary metabolites in the plant defences mechanism. SA was discovered to enhance phytoalexin production in cell culture and root culture of several medicinal plants such as *Withania somnifera, Anisodus luridus* and *Lepechinia caulescens* (Sivanandhan *et al.*, 2013; Qin *et al.*, 2014; Vergara Martínez *et al.*, 2017). However, SA not stimulate the double production both hypericin and pseudohypericin in callus and shoot culture of *Hypericum perforatum* but only in suspension cultures (Gadzovska *et al.*, 2013).

Since MeJA could give a detrimental effect in plant growth in the process of producing secondary metabolites in *Panax ginseng* (Kim *et al.*, 2004) and SA plays a significant role in modulating the cell redox status and defending plants against oxidative damage, the synergism of these elicitor were recently used in most plants. Recently, the enhancement of phenolic compounds in plant cell suspension cultures of *Thevetia peruviana* through synegistic effect of methyl jasmonate and salicylic acid were conducted by (Mendoza *et al.*, 2018). Synergistic effect of other elicitors on compounds accumulation was also reported by Gadzovska *et al.* (2013).

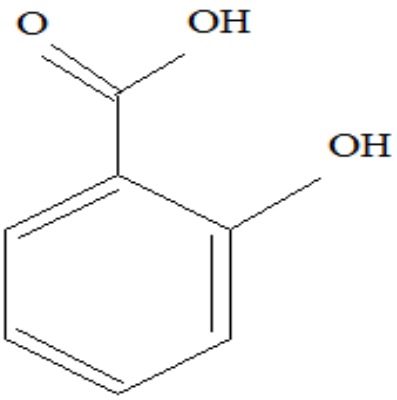

Figure 2.7: Chemical structure of salicyclic acid.

2.7 High Scale Production

2.7.1 Bioreactor

Bioreactors refers to the vessels/tank which provide the optimum condition that enable the fermentation and biochemical reactions of microorganisms to produce the desired products in a large scale. Appropriate bioreactors usually capable of sustaining the required biological functions by regulating temperature, pH, fluid velocity, shear stress, weight and heat transfer, O_2, CO_2 and nutrient supply, reaction rate and cell production (Zhong, 2011). This technology has led to remarkable achievements in the biotechnology field and have been used in many sectors such as wastewater treatment in the environmental protection industry, cell culture and tissue engineering in the healthcare sector, the manufacture of high-value pharmaceuticals and high-scale chemicals production industrial biotechnology (Li *et al.*, 2016; Pirasaci *et al.*, 2017; Zhuo *et al.*, 2018; Christoffersson & Mandenius, 2019). Besides, this system can be applied to all types of bio-catalysis including enzymes, cellular organelles, animals and plant cells.

In this modern days, scientist had been interested in exploiting plant cells as the source for plant-based drug production (Veeresham, 2012) and adventitious root culture had been widely used to serve the purpose because of their rapid growth as well as stable for bioactive compound production as demonstrated by Jalil *et al.* (2015). In addition, Pinostrobin extracted from the adventitious root cultures of *Boesenbergia rotunda* has anti-HIV, anti-ulcer, anti-leukaemia and anti-inflammatory activity (Yusuf *et al.*, 2018).

Extensive studies had been done on plant bioreactor since bioreactors offers a low-cost and automated system for commercial *in vitro* plant propagation and low cost secondary metabolite production. Several factors, as well as optimization techniques to improve their bio-catalysis efficiency, such as modification of inoculum density, bioreactor modification (size, agitation, and aeration), temperature and light exposure, have to be effectively addressed before using bioreactors. Table 2.2 depicted small-scale production of secondary metabolites with their optimized condition while using bioreactors.

Table 2.2: Production of secondary metabolites with their optimized bioreactors requirements.

Plant Species	Culture Type	Bioreactor Volume and Type	Operational Conditions	Metabolite Production, mgL^{-1}	References
Vitis vinifera* cv. Chasselas and *Vitis berlandieri	Suspension	Stirred (2-L)	Temperature :23 C Light regime: dark Agitation:50 rpm Flow rate: 0.025 vvm	Resveratrol 209	Donnez *et al.*, 2011
Bupleurum falcatum L.	Adventitious roots	Bubble Column Airlift (20-L)	Temperature :23 C Light regime: 12:12 light:dark Agitation:50 rpm Flow rate: 0.15 vvm	Saikosaponin-a, Saikosaponin-d, 500-600.	Kusakari *et al.*, 2012
***Salvia ocinalis* L**	Hairy roots Shoots	Nutrient Sprinkler (5-L)	Temperature :26 C Light regime: dark Pump:40s/50s breaks	Rosmarinic acid 477.13	Grzegorczyk *et al.*, 2010.
Dracocephalum Forrestii W.W. Smith	Shoots	Nutrient Sprinkler (10-L)	Temperature :26 C Light regime: 16:8 light:dark Pump:25s/2.5s breaks	Rosmarinic acid 38.26	Weremczuk-Je˙zyna *et al.*, 2019
***Leunorus sibiricus* L.**	Hairy roots	Nutrient Sprinkler (5-L)	Temperature :26 C Pump:40s/1.5s breaks	Rosmarinic acid 448	Sitarek *et al.*, 2018
Eleutherococcus koreanum Nakai	Adventitious roots	Bubble Column Airlift (3-L)	Temperature :22 C Light regime: dark Flow rate: 0.1 vvm 22	Rosmarinic acid 78.22	Lee *et al.*, 2015
Dracocephalum Forrestii W.W. Smith	Shoots	Nutrient sprinkler (10-L)	Temperature :26 C Light regime: 16:8 light:dark Pump:25s/2.5s breaks	Caffeic acid 0.07	Weremczuk-Je˙zyna *et al.*, 2019
***Leunorus sibiricus* L.**	Hairy roots	Nutrient Sprinkler (5-L)	Temperature :26 C Pump:40s/1.5s breaks	Caffeic acid 302	Sitarek *et al.*, 2018
Harpagophytum procumbens	Suspension	Stirred (3-L)	Temperature :26 C Light regime: dark Agitation:100 rpm Flow rate: $1L^{-1}$ min	Verbascoside 445.44	Georgiev *et al.*, 2011
		Bubble Column Airlift (1-L)	Temperature :26 C Light regime: dark Agitation:100 rpm Flow rate: $1L^{-1}$ min every 2 s	Verbascoside 496.30	

Comprehensive bioreactor operating system ensures optimum development of biomass and metabolites by maintaining low stress for cultivated plant cells, tissues or organs by adequate supply of oxygen and nutrients and uniform distribution of cultured biomass (Steingroewer *et al.* 2013). Recently, a large scale propagation on sugarcane, *Saccharum officinarum* L. were conducted by using bioreactors (Da silva *et al.*, 2020), *Vaccinium vitis* (Arigundam *et al.*, 2020) and *Myrtus communis* L. (Aka kacar *et al.*, 2020). Meanwhile, a large scale production of coumestrol (soybean isoflavonoids) from *Glycine max* (Lee *et al.*, 2019), galanthamine and amaryllidaceae alkaloids from *Leucojum aestivum* L. using various type of bioreactors (Ivanov *et al.*, 2020; Ptak *et al.*, 2020). There are many types of bioreactors using plant cell and tissue cultures with their advantages and disadvantages such as stirred bioreactor, bubble column reactor, airlift reactor, wave reactor, spray bioreactor, mist bioreactor and undertow bioreactor slug bubble bioreactor (Table 2.3).

Table 2.3: Several types of bioreactors with their advantages and disadvantages.

Types	Aeration	Advantages	Disadvantages	Uses
Stirred bioreactor	Bubbles/ airlift	Easy scale up Simple Well known	Shear stress Mechanic stress Foam	Suspensions
Bubble Column Airlift	Bubbles/ airlift	Low cost High efficient mass transfer	Viscosity Foam Shear stress	Immobilized Suspensions
Temporary immersion system (RITA)	Bubbles	Simple, reduction in asphyxiation Well known.	Low culture Volume	Somatic embryogenic cell
Wave	Bubbles	Bubble free surface aeration. Well investigated Uniformity of energy negligible foaming.	Rheological Issues of plant culture	Suspension and Immobilized cells

However, there are few limitations on using bioreactors despite optimizing the physical and chemicals requirements for the cells optimum growth including high concentrations of sugar in the medium, excessive foaming resulting from extracellular polysaccharides, fatty acids, contamination and sheer stress (Su *et al.*, 2019). Nevertheless, only a few cell culture-induced secondary metabolites were successfully commercialized by industries namely shikonin and barberine from *Lithospermum erythrorhizon* and *Camellia japonica* respectively 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.*, 2012).

For adventitious root cultures, there are several reports on the enhancement of bioactive compounds using balloon type bubble column bioreactor (BTBCB) i.e. production of gingsenosides from adventitious roots of *Panax ginseng* (Sivakumar *et al.*, 2005; Cao *et al*., 2020), biomass and bioactive compounds in adventitious roots of *Morinda citrifolia* (Baque et. al, 2014), *Eleutherococcus koreanum* (Lee *et al.*, 2015) and *Polygonum multiflorum* (Lee *et al.*, 2015) using balloon type airlift bioreactor.

CHAPTER 3: MATERIALS AND METHODS

3.1 Plant materials

Mature rhizomes of *Zingiber zerumbet* L. Smith were collected from nursery at Pusat Asasi Sains, University of Malaya and were cleaned thoroughly by washing repeatedly with tap water and maintained in the laboratory for shoot buds sprouting as in Figure 3.1

Figure 3.1: Shoot buds sprouting from the rhizome. *Bar: 2 cm*

3.2 Plant tissue culture

3.2.1 Explant sterilization

Young buds at 1.5-2.0 cm in length were used as explants and were surfaced sterilized with 70 % (v/v) Clorox (5.25% of sodium hypochlorite) solution with a few drops of Tween 20 to enhance the ability of the bleach to penetrate the plant to fully sterilize the sample. After 30 minutes, the roots and outer layer of shoot buds were removed and transferred aseptically into 30% (v/v) Clorox for 10 minutes. Subsequently, the shoot buds were rinsed with sterile distilled water and air-dried in the laminar air flow chamber.

3.2.2 Induction of adventitious roots of *Z. zerumbet*

The sterilized shoot buds were then excised to 1.0 cm in length and inoculated into Murashige and Skoog (1962) (MS) medium supplemented with different combination concentration of Napthalene-acetic-acid (NAA) and Indole-3-butryic acid (IBA) in the range of 0.5, 1 and 2 mg L^{-1}, 3% sucrose and solidified with 2 gL^{-1} gelrite. Roots grown in a medium without hormone served as control. The media pH was adjusted to 5.7 prior to autoclaving. The root cultures were maintained at 25 ± 1°C under light regime 16:8h (light: dark) and dark condition. For each treatment, percentage of root response, root length and number of root per explant were measured. All experiments were carried out in triplicate cultures with 5 explants in each replicate.

3.2.3 Combined effect of auxin and cytokinin on adventitious root growth and secondary metabolite production of *Z. zerumbet* adventitious root culture

After one month of induction, the induced roots were transferred into shake flask containing different media formulation. The roots were cultured in MS media with different combination of auxin-auxin and auxin-cytokinin supplemented with 3% sucrose. The PGR involved were 6-Benzylaminopurine (BAP) and Indole-3-butyric acid (IBA) in the range of 1, 3, 5 and 7 mg L^{-1}. Roots grown in a medium containing 1mgL^{-1} NAA served as control. The media pH was adjusted to 5.7 prior to autoclaving. The root cultures were maintained at 25 ± 1°C under light regime 16:8h (light: dark) and dark condition. For each treatment, fresh weight (FW) and dry weight (DW) were measured. Measurement of fresh and dry biomass was made at 6 intervals during 30 days of culture. The growth parameters namely the specific growth rate (μ) and doubling time (td) were calculated for the adventitious root liquid culture according to the following equation

(Loyola-Vargas and V á zquez-Flota, 2006). All experiments were carried out in triplicate cultures and each treatment was repeated twice.

Equation 1:

$$\ln x = \mu t + \ln x_0$$

$$\mu = \frac{\ln x - \ln x_0}{t}$$

Where;

μ= Specific growth rate
X_0= Initial fresh weight
X= Final fresh weight after incubation
t= Days of incubation

Equation 2:

$$td = \frac{\ln 2}{\mu}$$

Where;

td= Doubling time
μ= Specific growth rate

3.2.4 Determination of root biomass

Roots were collected after 30 days of incubation for each treatment and washed with distilled water. The fresh roots were pressed gently on filter paper (Whatman Ltd., England) to remove excess water and weighed for the fresh weight (g). The roots were dried at 60°C until a consistent weight were obtained to get the dry weight (Baque *et al.,* 2013). The data were presented as FW (g) and DW (g).

3.3 Compound Extraction and Identification

3.3.1 Soxhlet extraction

The dried root were pulverized to powder and was weight about 0.1 g. The grounded sample were inserted into the cellulose extraction thimble. Soxhlet extraction includes the condenser, water bath, cellulose extraction thimble and round flask was set as Figure 3.2. The boiling point of water bath was set to 39°C similar to the boiling point of Dichloromethane (Merck, USA) and the condenser was set to 13-14°C for a period of 6 hours (Jalil *et al.,* 2018).

Figure 3.2: Soxhlet extraction

3.3.2 HPLC setup and solvent gradient configuration

The Shimadzu HPLC system comprises of a SIL-20A HT auto sampler, LC-20 AT multi-solvent delivery system, DGU-20A degasser, CTO-20 AC cooler and SPD-M20A uv/visible detector (Figure 3.3). The device was operated by using LC Solution software. The reverse column and guard (Chromolith $RP^{-1}8$ encapped, 100-4.6 mm) used were from Merck, US. The solvent used were methanol grade HPLC (Merck Ltd.), acetonitrile grade HPLC (Fisher Ltd.), phosphoric acid grade HPLC (BDH Ltd.) and ultrapure water with 0.1% phosphoric acid. The column and guard column were cleaned with pure HPLC grade acetonitrile before and after usage.

Figure 3.3: Shimadzu HPLC system

3.3.3 Identification of zerumbone and α--humulene

The zerumbone and α-humulene compounds were classified by comparing the retention times from sample to a standards purchased from Sigma (USA). The extract was spiked with a combination of standards when there was ambiguity. The compound were classified by comparing the retention times, spectral features and intensities of the peaks of the chromatograms obtained with and without spiking of the samples. The amounts of the compounds were calculated as follows:

Equation 2:

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

3.3.4 Standard calibration curve for α--humulene and zerumbone

The standard α-humulene and zerumbone from Sigma (USA) was used as standards. The retention time of the standards was determined from HPLC chromatogram. The solvent used to prepare all the solutions was acetonitrile (ACN) with concentration 0.05, 0.1, 0.2, 0.5 and 1 mg/ml, flow rate of 1 µL/min, temperature of 27°C and running time of 15 minutes. Three replicates were prepared for each concentration and the injection was also done in triplicate. The peak area of each dilution for each standard compound was calculated from the HPLC chromatograms and a standard calibration was obtained.

3.4 Elicitation of adventitious root cultures

3.4.1 Synergistic effect of methyl jasmonate and salicyclic acid on cell growth and bioactive compounds production

Elicitation was done by combining methyl jasmonate (MeJA) and salicyclic acid **(**SA) at different concentrations. The combination treatments are as shown in Table 3.1 were introduced at day 15 of cultivation for 26 days (Jalil *et al.,* 2018). Adventitious root culture without elicitation was used as control harvested after 26 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, equivalent volume of sterilized distilled water was added to the cultures as blank treatment control for the different elicitor concentrations studied. All experiments was done in triplicate cultures and each treatment was repeated three times.

Table 3.1: Combination concentration of elicitors (Methyl Jasmonate and Salicyclic Acid).

Treatment	Concentration MeJA (µM)	Concentration SA (µM)
1	400	400
2	400	600
3	400	800
4	400	1200
5	600	400
6	600	600
7	600	800
8	600	1200
9	800	400
10	800	600
11	800	800
12	800	1200
13	1200	400
14	1200	600
15	1200	800
16	1200	1200

3.5 Scale up of adventitious root cultures using bioreactor

3.5.1 Bioreactor system establishment for adventitious root cultures

Up scaling of adventitious root cultures was done in bioreactor as shown in Figure 3.4. Root cultures aged one month old with initial inoculum density of 10g FW was cultured in a five-liters (5 L) balloon type bubble column bioreactor (BTBCB) at 1.0 L/min air flow at 25 ± 1°C. The pH of the medium was kept constant at pH 5.7 and automatically controlled using an automated controller fabricated by Fermentec Resources Sdn. Bhd. The working volume was one-liter and the roots was cultivated in an optimized media obtained from shake flask system (media supplemented with 1 mgL^{-1} NAA and 3 mgL^{-1} BAP at 16:08 light regime). The roots was collected after 30 days of cultivation for bioactive compounds determination. The FW and final dry weight (DW) was recorded. All experiments were carried out in triplicate cultures and were repeated twice.

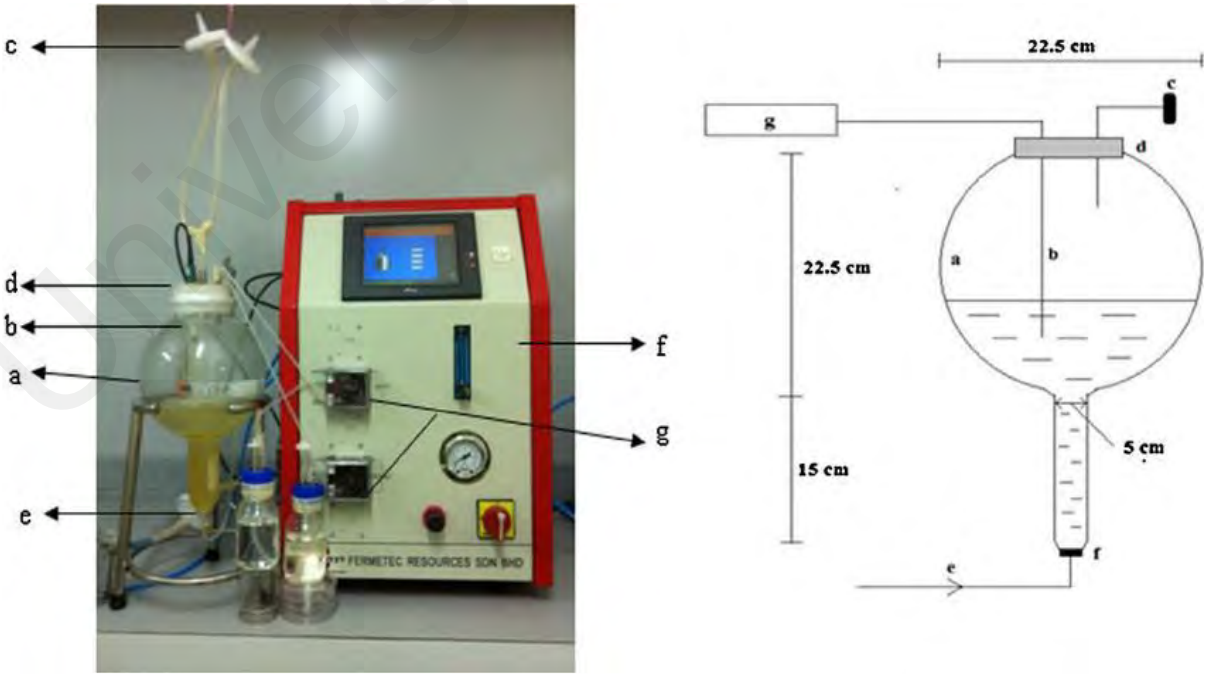

Figure 3.4: Configuration of 5-L balloon type bubble column bioreactor (BTBCB), (a) Balloon glass tank (b) pH meter, (c) air filter, (d) silicon cap, (e) filtered air inlet (f) gas flow rate float meter and (g) peristaltic pump for acid/base addition. Source: Chin *et al.*, 2014

CHAPTER 4: RESULTS

4.1 Establishment of adventitious root culture of *Z. zerumbet*

4.1.1 Initiation of adventitious root culture

Root induction time was varied with concentration and different combinations of PGR. The first adventitious root formation was observed after seven days of inoculation in MS media supplemented with 1.0 mgL^{-1} NAA and 2.0 mgL^{-1} IBA and incubated in dark condition. Even though media supplemented with 1.0 mgL^{-1} NAA and 1.0 mgL^{-1} IBA produced significantly highest number of root per explant (18.0 ± 1.0) with rooting percentage of 93 %, media supplemented with 1.0 mgL^{-1} NAA and 2.0 mgL^{-1} IBA produced the highest number of root responses with full percentage (100 %), and the numerous roots formed was highly branched with significantly maximum length of 7.3 ± 1.3 cm as shown in Table 4.1. In order to obtain the maximum number of adventitious roots with high potential for biomass and secondary metabolites production, media supplemented with 1.0 mgL^{-1} NAA and 2.0 mgL^{-1} IBA was selected for further root suspension experiments.

The lowest percentage of root response was 13 % with root length 0.4 ± 0.1 cm observed in control (untreated media) without supplementation of PGRs besides delayed formation at 14 days. Lower percentage of root response (20%) was also observed in the media with lowest concentration of NAA and IBA albeit longer root length (0.8 ± 0.4 cm) as compared to control (Table 4.1). Meanwhile, higher concentration of both NAA and IBA in the media resulted in the root response of 87 % whilst comparable root length at 1.0 ± 0.6 cm.

Table 4.1: Initiation of adventitious roots of *Z. zerumbet* in MS media supplemented with different combination and concentration of plant growth regulator after four weeks induction in dark condition.

Plant growth regulators (mgL^{-1})	Percentage (%)	Root length (cm)	Root per explant
Control	13	0.4 ± 0.1^{e}	2.0 ± 0.6^{e}
0.5 NAA + 0.5 IBA	20	0.8 ± 0.4^{ed}	1.6 ± 1.2^{ef}
0.5 NAA + 1.0 IBA	60	0.3 ± 0.1^{e}	4.7 ± 1.5^{cde}
0.5 NAA + 2.0 IBA	67	2.1 ± 0.5^{bcde}	6.7 ± 2.1^{c}
1.0 NAA + 0.5 IBA	47	1.7 ± 0.3^{bcde}	4.7 ± 3.1^{cde}
1.0 NAA + 1.0 IBA	93	3.3 ± 0.7^{b}	18.0 ± 1.0^{a}
1.0 NAA + 2.0 IBA	100	7.3 ± 1.3^{a}	11.7 ± 1.5^{b}
2.0 NAA + 0.5 IBA	40	2.6 ± 0.8^{bc}	5.7 ± 2.1^{cd}
2.0 NAA + 1.0 IBA	73	2.2 ± 0.3^{bcd}	6.3 ± 1.5^{c}
2.0 NAA + 2.0 IBA	87	1.0 ± 0.6^{cde}	5.3 ± 2.1^{cd}

Means with different letters in the same column are significantly different at $p < 0.05$ according to ANOVA and Tukey's multiple range test

4.1.2 Multiplication of adventitious root culture in shake-flask system

Establishment of adventitious root culture (AdRC) from *Z. zerumbet* (Figure 4.1) was done in MS media with different concentrations of IBA and BAP supplemented with 1 mgL^{-1} NAA in shake flask.

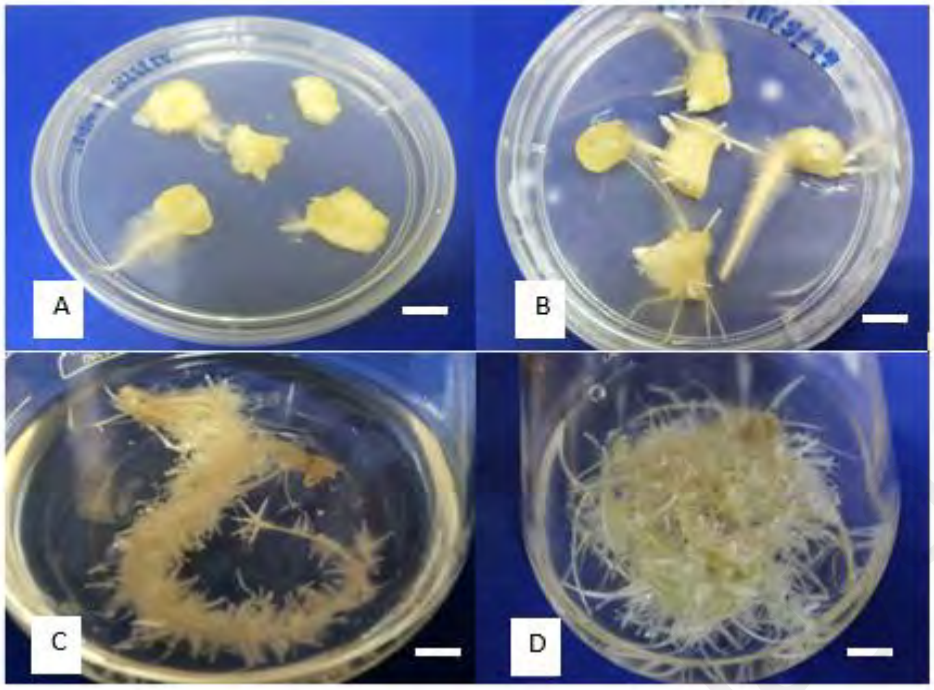

Figure 4.1: Establishment of adventitious root culture of *Z. zerumbet* *A*: Root induction in control treatment after 14 days of inoculation *B*: Root induction in MS media supplemented with 1 mgL^{-1} NAA and 2 mgL^{-1} IBA after 7 days of inoculation *C*: Multiplication of adventitious root culture in MS media supplemented with 1 mgL^{-1} NAA and 3 mgL^{-1} BAP after five days in shake flask *D*: Multiplication of adventitious root culture in MS media supplemented with 1 mgL^{-1} NAA and 3 mgL^{-1} BAP after 15 days in shake flask. *Bar: 0.5 cm*

The maximum fresh weight (6.9 ± 0.1 g FW) and dry weight (2.1 g DW) with highest specific growth rate and doubling time were obtained at 1 mgL^{-1} NAA supplemented with 5 mgL^{-1} IBA in 16:08 photoperiod (Table 4.2). However, this treatment showed insignificant difference with media 1 mgL^{-1} NAA supplemented with 3 mgL^{-1} BAP. Meanwhile, treatment with 1 mgL^{-1} NAA supplemented with 5 mg/ L BAP also showed insignificant difference with treatment 1 mgL^{-1} NAA supplemented with 7 mgL^{-1} BAP in 16:08 photoperiod with specific growth rate ranging around 0.5 ± 0.02 day^{-1} to 0.7 ± 0.04 day^{-1}. Treatment with lower concentration of IBA (1 mgL^{-1}) showed significant difference in 16:08 photoperiod with lower specific growth rate 0.8 ± 0.02 day^{-1} compared to control. No significant difference was observed for incubation in dark condition. In contrast, treatment with lower concentration of BAP (1 mgL^{-1}) showed significant increase in FW (5.9 ± 0.08 g and 6.2 ± 0.04 g) and specific growth rate (1.9 ± 0.02 day^{-1} and 1.9 ± 0.03

day^{-1}) in both light regimes. The lowest multiplication of adventitious roots was observed at the highest concentration of IBA and BAP (7 mgL^{-1}) with lowest specific growth rate and doubling time for both 16:08 photoperiod and dark condition (Table 4.2). Figure 4.2-4.5 shows pattern of root growth incubated in combined auxin-auxin (NAA + IBA) media and auxin-cytokinin (NAA + BAP) media (dark and16:08 photoperiod).

Table 4.2: Combined effects of auxin-auxin and auxin-cytokinin on adventitious root growth of *Z. zerumbet* in 16:08 photoperiod and dark condition after 30 days of cultivation.

Light regime	PGR	Treatment	Concentration (mgL^{-1})	Fresh weight (g)	Dry weight (g)	Specific growth rate(μ)
16:08	Control (NAA)	Control 16:08	1	3.90± 0.03^{e}	1.20 ± 0.03^{b}	1.40±0.03^{c}
	Control + IBA	A	1	2.00 ±0.07^{h}	0.60±0.03^{c}	0.80±0.02^{d}
		B	3	3.00±0.06^{g}	0.90±0.02^{bc}	1.20±0.02^{cd}
		C	5	6.90±0.08^{a}	2.10±0.01^{a}	2.10±0.01^{a}
		D	7	1.90±0.06^{i}	0.60±0.03^{c}	0.70±0.03^{d}
	Control + BAP	E	1	5.90±0.08^{c}	1.80±0.02^{ab}	1.90±0.02^{a}
		F	3	6.80±0.05^{a}	1.90±0.05^{a}	2.00±0.05^{a}
		G	5	1.80±0.13^{i}	0.50±0.04^{c}	0.70±0.04^{d}
		H	7	1.60±0.07^{i}	0.40±0.02^{c}	0.50±0.02^{e}
Dark	Control (NAA)	Control dark	1	3.60 ±0.05^{f}	1.10±0.02^{b}	1.40±0.01^{c}
	Control + IBA	I	1	3.60± 0.13^{f}	1.10±0.04^{b}	1.40±0.03^{c}
		J	3	6.30±0.06^{bc}	1.90±0.03^{a}	1.90±0.03^{a}
		K	5	6.70±0.07^{a}	2.00±0.04^{a}	2.00±0.03^{a}
		L	7	3.40±0.07^{fg}	1.00±0.02^{b}	1.30±0.01^{c}
	Control + BAP	M	1	6.20±0.04^{bc}	1.90±0.04^{a}	1.90±0.03^{a}
		N	3	6.40±0.08^{b}	1.90±0.03^{a}	1.90±0.02^{a}
		O	5	4.40±0.10^{d}	1.30±0.03^{b}	1.60±0.02^{b}
		P	7	2.40±0.05^{h}	0.80±0.03^{c}	1.00±0.03^{cd}

Means with different letters in the same column are significantly different at $p < 0.05$ according to ANOVA and Tukey's multiple range test

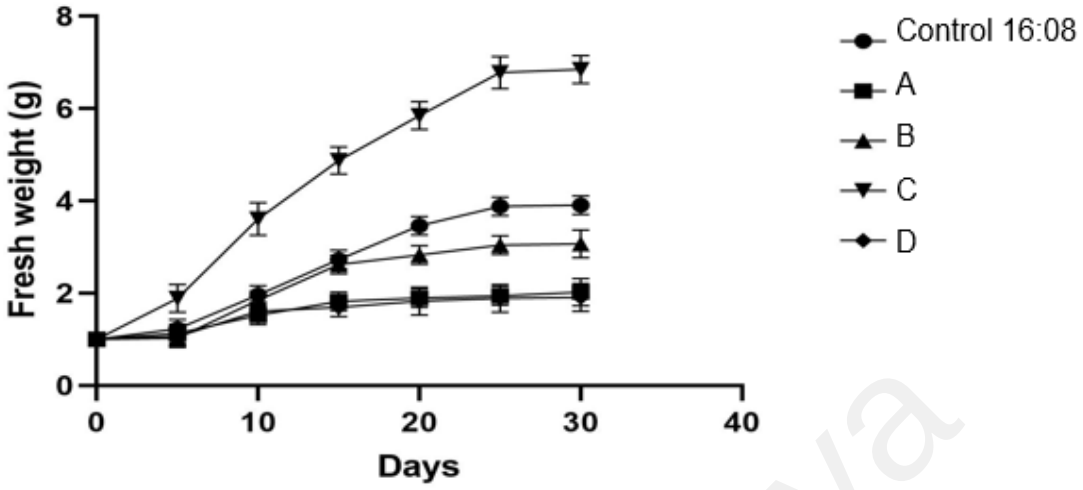

Figure 4.2: Root growth in combined auxin-auxin (NAA + IBA) media in 16:08 photoperiod. *A*: MS media supplemented with 1 mgL^{-1} NAA and 1 mgL^{-1} IBA; *B*: MS media supplemented with 1 mgL^{-1} NAA and 3 mgL^{-1} IBA; *C*: MS media supplemented with 1 mgL^{-1} NAA and 5 mgL^{-1} IBA; *D*: MS media supplemented with 1 mgL^{-1} NAA and 7 mgL^{-1} BAP. Error bar indicates standard deviation of the mean value.

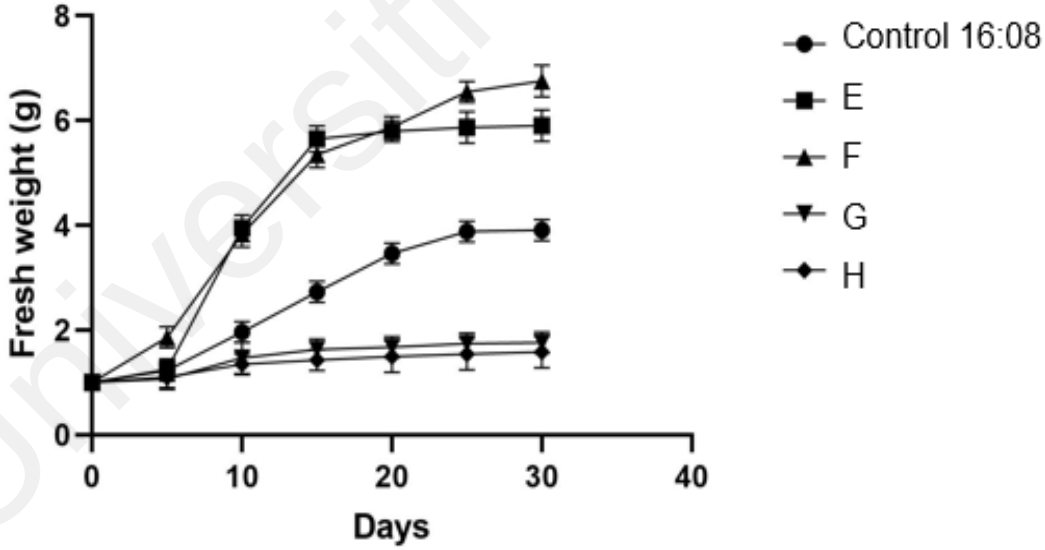

Figure 4.3: Root growth in combined auxin-cytokinin (NAA + BAP) media in 16:08 photoperiod. *E*: MS media supplemented with 1 mgL^{-1} NAA and 1 mgL^{-1} BAP; *F*: MS media supplemented with 1 mgL^{-1} NAA and 3 mgL^{-1} BAP; *G*: MS media supplemented with 1 mgL^{-1} NAA and 5 mgL^{-1} BAP; *H*: MS media supplemented with 1 mgL^{-1} NAA and 7 mgL^{-1} BAP. Error bar indicates standard deviation of the mean value.

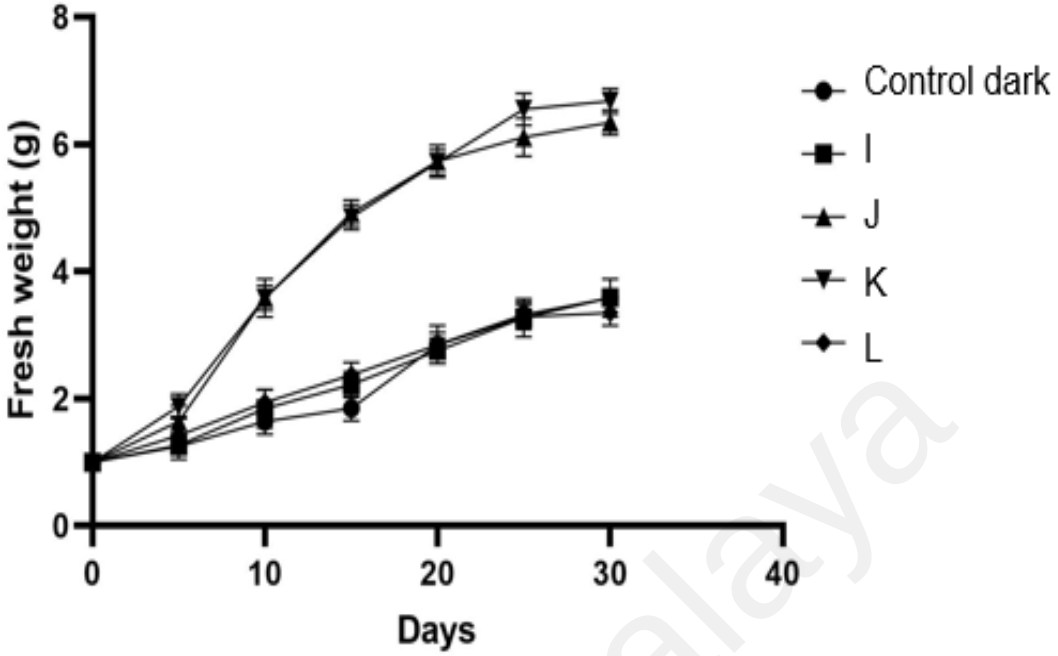

Figure 4.4: Root growth in combined auxin-auxin (NAA + IBA) media in dark condition. *I*: MS media supplemented with 1 mgL^{-1} NAA and 1 mgL^{-1} IBA; *J*: MS media supplemented with 1 mgL^{-1} NAA and 3 mgL^{-1} IBA; *K*: MS media supplemented with 1 mgL^{-1} NAA and 5 mgL^{-1} IBA; *L*: MS media supplemented with 1 mgL^{-1} NAA and 7 mgL^{-1} BAP. Error bar indicates standard deviation of the mean value.

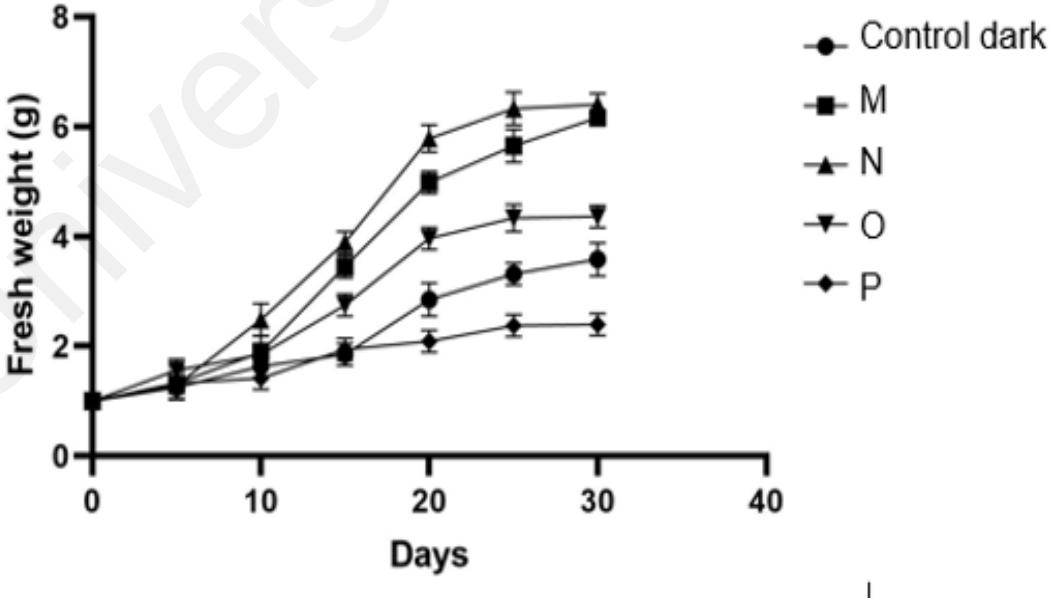

Figure 4.5: Root growth in combined auxin-cytokinin (NAA + BAP) media in dark condition. *M*: MS media supplemented with 1 mgL^{-1} NAA and 1 mgL^{-1} BAP; *N*: MS media supplemented with 1 mgL^{-1} NAA and 3 mgL^{-1} BAP; *O*: MS media supplemented with 1 mgL^{-1} NAA and 5 mgL^{-1} BAP; *P*: MS media supplemented with 1 mgL^{-1} NAA and 7 mgL^{-1} BAP. Error bar indicates standard deviation of the mean value.

4.2 Establishment of standard calibration for α-humulene and zerumbone

Investigation to determine suitable solvent system, elution gradient and resolution efficiency to elute α-humulene and zerumbone was carried out for HPLC analysis. α-humulene is strongly detected at λ= 200 nm and low signal of detection at λ= 254 nm. Conversely, zerumbone shows strong signal at λ= 254 nm and weaker at λ= 200 nm. Nevertheless, both compounds showed optimum absorption at λ =243 nm, which was applied as detection wavelength for both compounds.

The representative chromatograms for standards are shown in Figures 4.6 - 4.8. The retention times for α-humulene and zerumbone are at 5.7 - 6.7 and 9.5 - 11.1 minutes respectively.

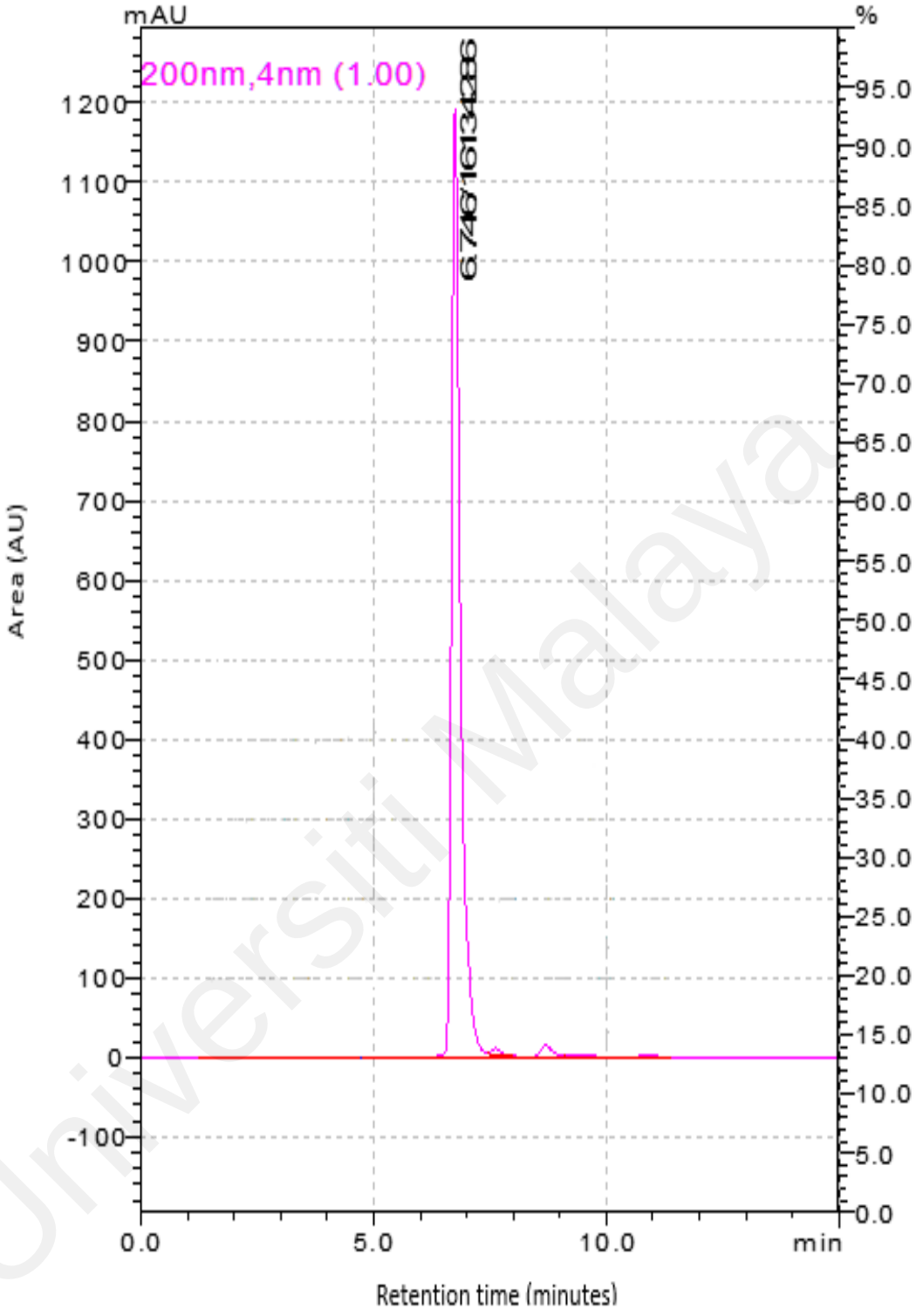

Figure 4.6: Chromatogram for standard α-humulene (Sigma, USA) at 200 nm.

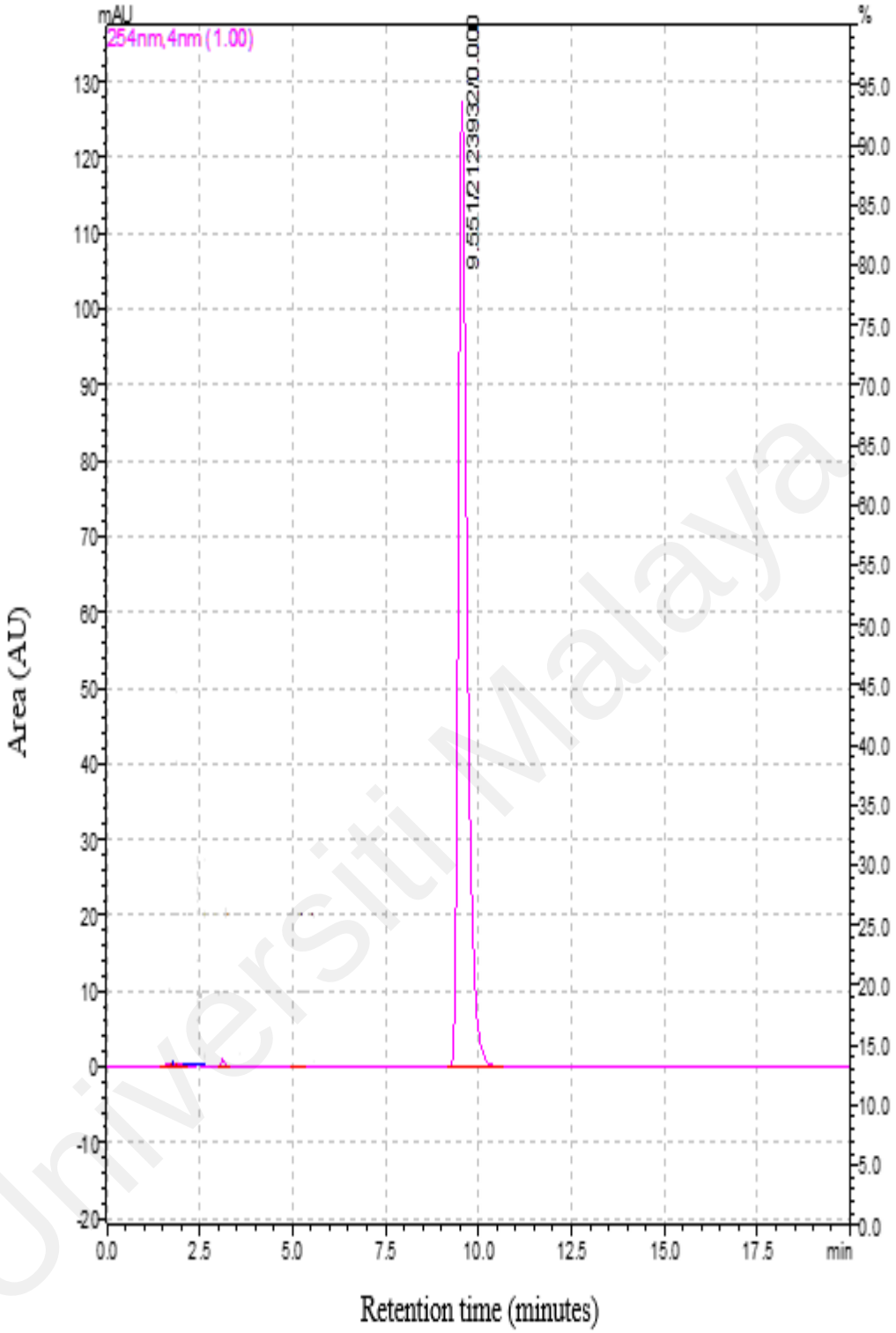

Figure 4.7: Chromatogram for standard zerumbone (Sigma, USA) at 254 nm.

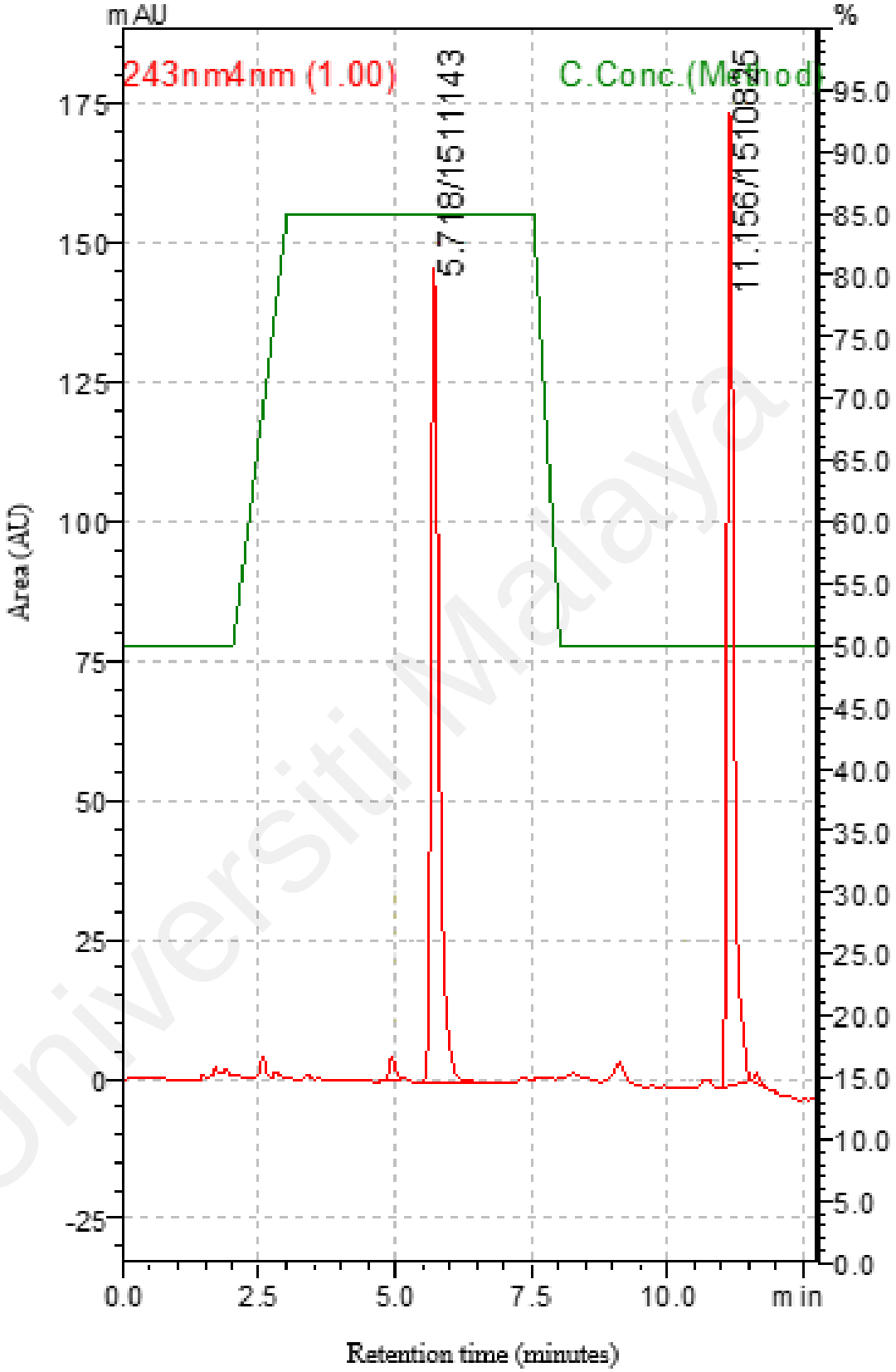

Figure 4.8: Chromatogram for a mixture of standard α-humulene (Sigma,USA) and zerumbone (Sigma,USA) at 243 nm.

Standard calibration for both compounds were carried out at λ = 243 nm. Figure 4.9 shows the α-humulene standard calibration with linear correlation coefficient, $R^2 = 0.999$; while Figure 4.10 shows zerumbone standard calibration with linear correlation coefficient, $R^2 = 0.999$.

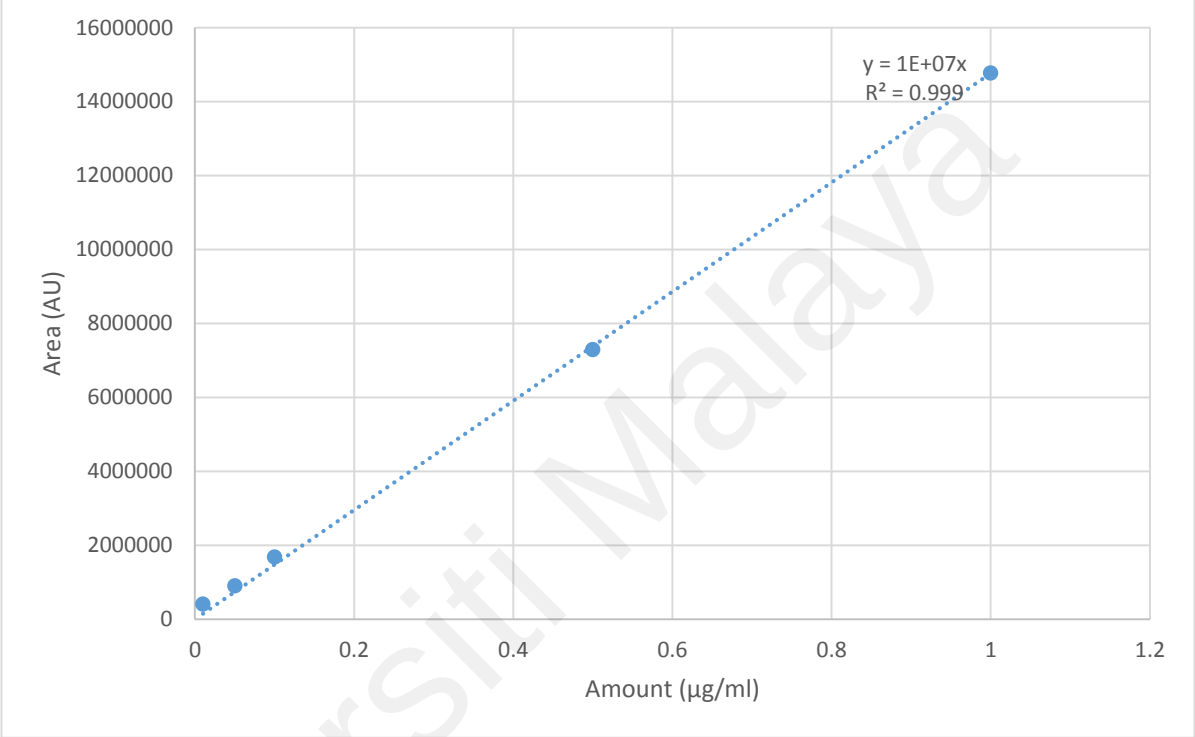

Figure 4.9: Standard calibration for α-humulene. (Sigma, USA)

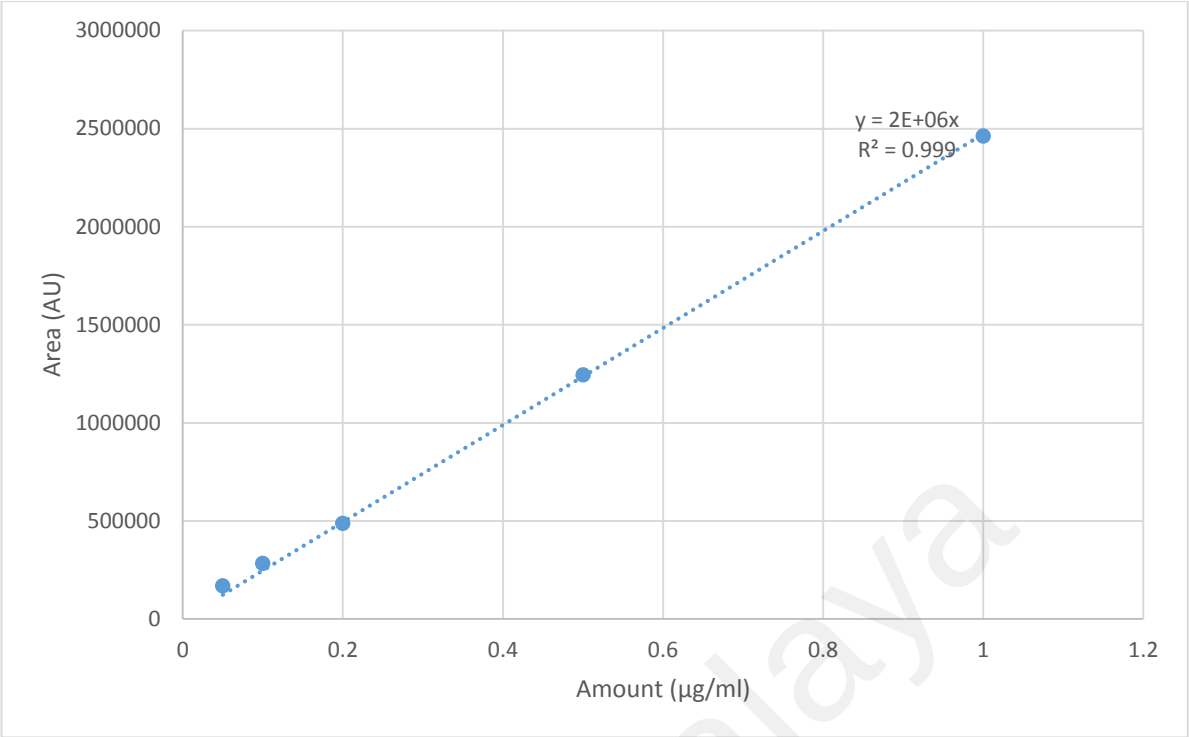

Figure 4.10: Standard calibration for zerumbone. (Sigma, USA)

4.3 Synergistic effects of plant growth regulators (PGR) on root biomass growth and secondary metabolite production

4.3.1 Combined effects of auxin-auxin (NAA and IBA)

Optimization of root using different concentrations and types of PGR was carried out in liquid media to produce a sustainable amount of root biomass and compounds for potential large-scale production. The amount of α-humulene and zerumbone were significantly higher at 16:08 photoperiod compared to dark condition in Figure 4.11.

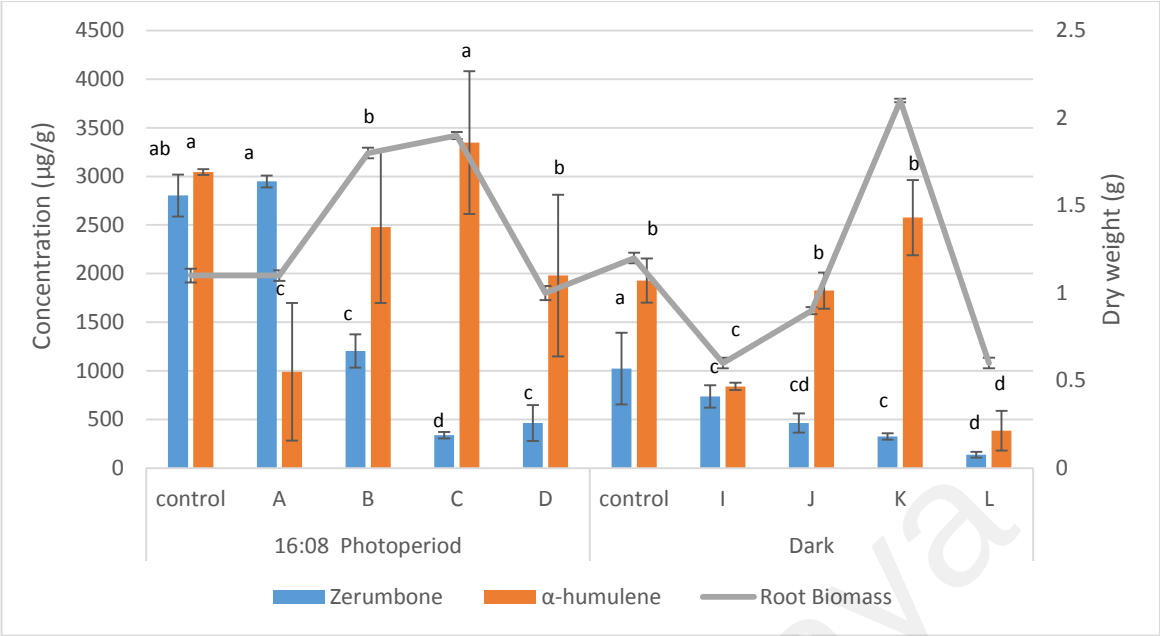

Figure 4.11: Zerumbone, α-humulene and root produced in combined auxin-auxin media at 16:08 photoperiod and dark condition. *A*: MS media supplemented with 1 mg/L NAA and 1 mg/L IBA *B*: MS media supplemented with 1 mg/L NAA and 3 mg/L IBA; *C*: MS media supplemented with 1 mg/L NAA and 5 mg/L IBA; *D*: MS media supplemented with 1 mg/L NAA and 7 mg/L BAP; *I*: MS media supplemented with 1 mg/L NAA and 1 mg/L IBA; *J*: MS media supplemented with 1 mg/L NAA and 3 mg/L IBA; *K*: MS media supplemented with 1 mg/L NAA and 5 mg/L IBA; *L*: MS media supplemented with 1 mg/L NAA and 7 mg/L BAP. Error bar indicates standard deviation of the mean value. Means with different letters in the same column are significantly different at $p < 0.05$ according to ANOVA and Tukey's multiple range test.

The highest amount of zerumbone (2984 ± 61 µg/g) was obtained in MS supplemented with 1 mgL^{-1} NAA and 1 mgL^{-1} IBA (Table 4.3) as shown in treatment A and the highest α-humulene (3547 ± 356 µg/g) was produced in MS supplemented with 1 mgL^{-1} NAA and 5 mgL^{-1} IBA (Table 4.3) as shown in treatment C propagated at 16:08 photoperiod. The highest root biomass (2.1 ± 0.1 g) were observed in media MS supplemented with 1 mgL^{-1} NAA and 5 mgL^{-1} IBA at dark condition but producing lower amount of zerumbone (325 ± 34 µg/g) and α-humulene (2576 ± 387 µg/g) (Table 4.3) as shown in treatment K (Figure 4.11).

Table 4.3: Synergistic effects of auxin-auxin on zerumbone, α-humulene and root biomass of *Z. zerumbet* in 16:08 photoperiod and dark condition after 30 days of culture.

Light regime	Concentration PGR (mg/L) Control + IBA	Zerumbone (µg/g)	α-humulene (µg/g)	Root Biomass (g)
Control (NAA)	1.0	2803 ± 216^{ab}	3046 ± 30^{a}	1.1 ± 0.02^{e}
16:08	1.0	2984 ± 61^{a}	989 ± 708^{c}	1.1 ± 0.04^{e}
	3.0	1204 ± 170^{c}	2888 ± 1958^{b}	1.9 ± 0.03^{c}
	5.0	338 ± 34^{d}	3547 ± 356^{a}	2.0 ± 0.04^{a}
	7.0	463 ± 183^{c}	1998 ± 1661^{b}	1.0 ± 0.02^{f}
Control (NAA)	1.0	1023 ± 368^{a}	1929 ± 227^{b}	1.2 ± 0.03^{e}
Dark	1.0	737 ± 115^{c}	840±39^{cd}	0.6±0.03^{e}
	3.0	463 ± 98^{cd}	1825±186^{bc}	0.9±0.02^{d}
	5.0	325±34^{c}	2576±387^{b}	2.1±0.01^{a}
	7.0	137±30^{d}	384±205^{d}	1.6±0.03^{e}

4.3.2 Combined effects of auxin-cytokinin (NAA and BAP)

Significant decrease in root biomass growth and compound production with increased concentration of BAP in both light regimes was observed (Figure 4.12). However, the amounts of zerumbone (3440 ± 168 µg/g) and α-humulene (3759 ± 798 µg/g) were highest in MS media supplemented with 1 mgL^{-1} NAA and 3 mgL^{-1} BAP at 16:08 photoperiod (Table 4.4) as shown in treatment F with 1.90 ± 0.1 g of root biomass (Figure 4.12). The lowest amounts of zerumbone (137 ± 7µg/g) and α-humulene (60 ± 22 µg/g) were observed in media containing highest BAP amount (7 mgL^{-1}) with dark condition; lowest root biomass amount 0.4 ± 0.02 g was also produced (Table 4.4) as shown in treatment P (Figure 4.12).

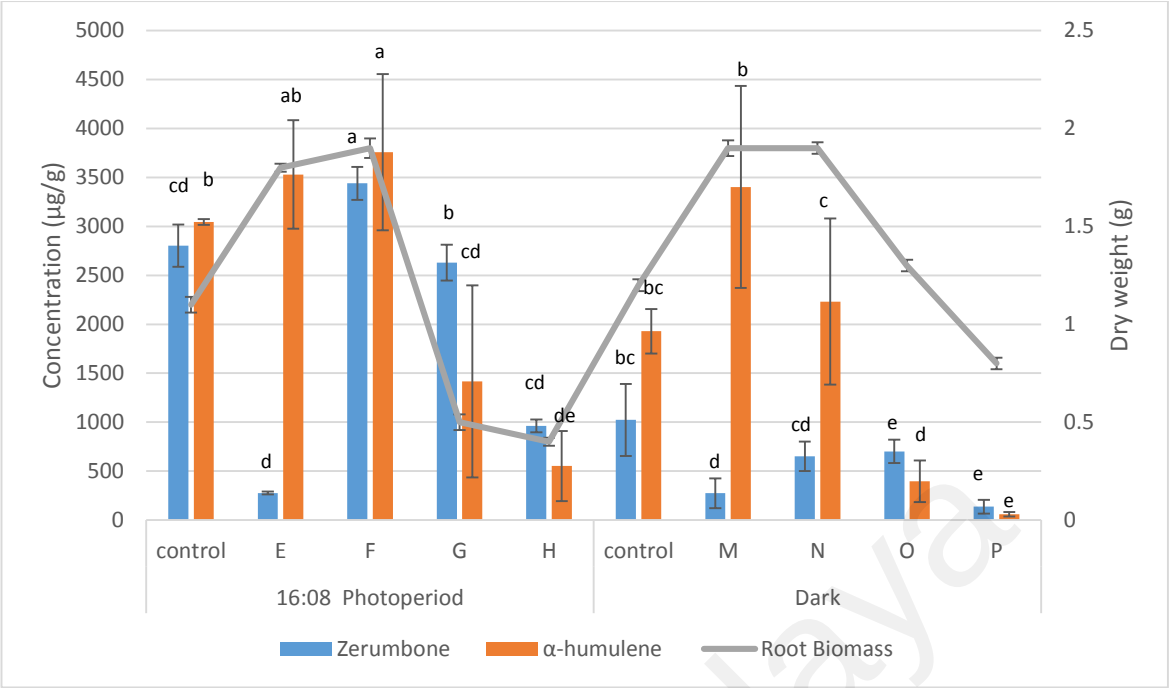

Figure 4.12: Zerumbone, α-humulene and root producedin combined auxin-cytokinin media at 16:08 photoperiod and dark condition. *E*: MS media supplemented with 1 mg/L NAA and 1 mg/L BAP; *F*: MS media supplemented with 1 mg/L NAA and 3 mg/L BAP; *G*: MS media supplemented with 1 mg/L NAA and 5 mg/L BAP; *H*: MS media supplemented with 1 mg/L NAA and 7 mg/L BAP; *M*: MS media supplemented with 1 mg/L NAA and 1 mg/L BAP; *N*: MS media supplemented with 1 mg/L NAA and 3 mg/L BAP; *O*:MS media supplemented with 1 mg/L NAA and 5 mg/L BAP; *P*:MS media supplemented with 1 mg/L NAA and 7 mg/L BAP. Error bar indicates standard deviation of the mean value. Means with different letters in the same column are significantly different at $p < 0.05$ according to ANOVA and Tukey's multiple range test.

Even though the amount of zerumbone was significantly lower at dark condition as compared to 16:08 photoperiod, the amount of α-humulene was less affected in both light regimes (Figure 4.12). Thus, to obtain significant increase in both secondary metabolites of interest and biomass production relative to control, root cultures were maintained in the optimized media (MS media supplemented with 1 mgL^{-1} NAA and 3 mgL^{-1} BAP) at 16:08 light regime for further elicitation experiments and larger volume production.

Table 4.4: Synergistic effects of auxin-cytokinin on zerumbone, α-humulene and root biomass of *Z. zerumbet* in 16:08 photoperiod and dark condition after 30 days of culture.

Light regime	Concentration PGR (mg/L) Control + BAP	Zerumbone (µg/g)	α-humulene (µg/g)	Root Biomass (g)
Control (NAA)	1.0	2803 ± 216^{cd}	3046 ± 30^{b}	1.1 ± 0.02^{d}
16:08	1.0	276±14^{d}	3964±3283^{ab}	1.8±0.02^{b}
	3.0	3440±168^{a}	3759±798^{a}	1.9±0.1^{a}
	5.0	2629±183^{b}	1416±1402^{cd}	0.5±0.04^{f}
	7.0	962±64^{cd}	60±22de	0.4±0.02^{g}
Control (NAA)	1.0	1023 ± 368^{bc}	1929 ± 227^{bc}	1.2 ± 0.03^{c}
Dark	1.0	274 ±151^{d}	3403±1031^{b}	1.9±0.04^{a}
	3.0	651±150^{cd}	2272±849^{c}	1.9±0.03^{a}
	5.0	701±120^{e}	395±213^{d}	1.3±0.03^{c}
	7.0	137±70^{e}	60±22^{e}	0.8±0.03^{e}

4.4 Synergistic effects of elicitors on root growth and secondary metabolite production

Z. zerumbet root cultures that had been established in the shake flask system was treated with different concentration and combination of methyl jasmonate (MeJA) and salicyclic acid (SA). It is hypothesized that applying the combination of the two elicitors will enhance the targeted compound production without affecting the root biomass growth. Figure 4.13 shows quantity of root biomass, zerumbone and α-humulene produced in the MeJA + SA elicited root cultures after 26 days of cultivation following elicitation. Root biomass decreased with increasing concentration of MeJA and SA as shown in Figure 4.13. When the roots were treated with MeJA and SA, root biomass production decreased by 36 % relative to control at T5 (600 µM MeJA + 400 µM SA),

whereas T15 (1200 µM MeJA + 800 µM SA) decreased root biomass production by 66 % compared to control (Table 4.5). The observed effects following treatments are probably due to stress response to the elicitors at aforementioned concentrations. Treatment with highest concentration of elicitors (1200 µM MeJA + 1200 µM SA) give lowest amount of root biomass production at 0.69 g i.e. relatively 76 % less after elicitation compared to control.

The combination of MeJA and SA exhibited less stress effect to the culture at lower concentrations since root biomass growth and compound production were not significantly affected. Although root biomass production decreased following treatment with elicitor, there is no significant difference determined when treated with combination of elicitors from 400 µM to 800 µM ranges. In this study, the amount of zerumbone was higher than α-humulene in all treated root cultures of *Z. zerumbet* as shown in Figure 4.13. It is suggested that combination of MeJA and SA encouraged the conversion of α-humulene thus assisted in the synthesis of zerumbone.

The combined application of MeJA and SA resulted in the highest production of zerumbone and α-humulene in adventitious root cultures of *Z. zerumbet* (Figure 4.13). Among the treatments, 400 µM MeJA + 400 µM SA yielded the highest improvement of zerumbone production (43.0 ± 0.2 x 10^3 µg/g) *viz*. 13-fold increase; treatment with 400 µM MeJA + 600 µM SA yielded highest α-humulene production (15.8 ± 5.1 x 10^3 µg/g) *viz*. 4.3-fold increase after 26 days of cultivation following elicitation compared to control. The synergism effect of MeJA and SA at 400 µM concentration with elicitation point at day 15 is concluded to be the most favourable treatment to produce significant amount of target compound *in vitro* (Table 4.5).

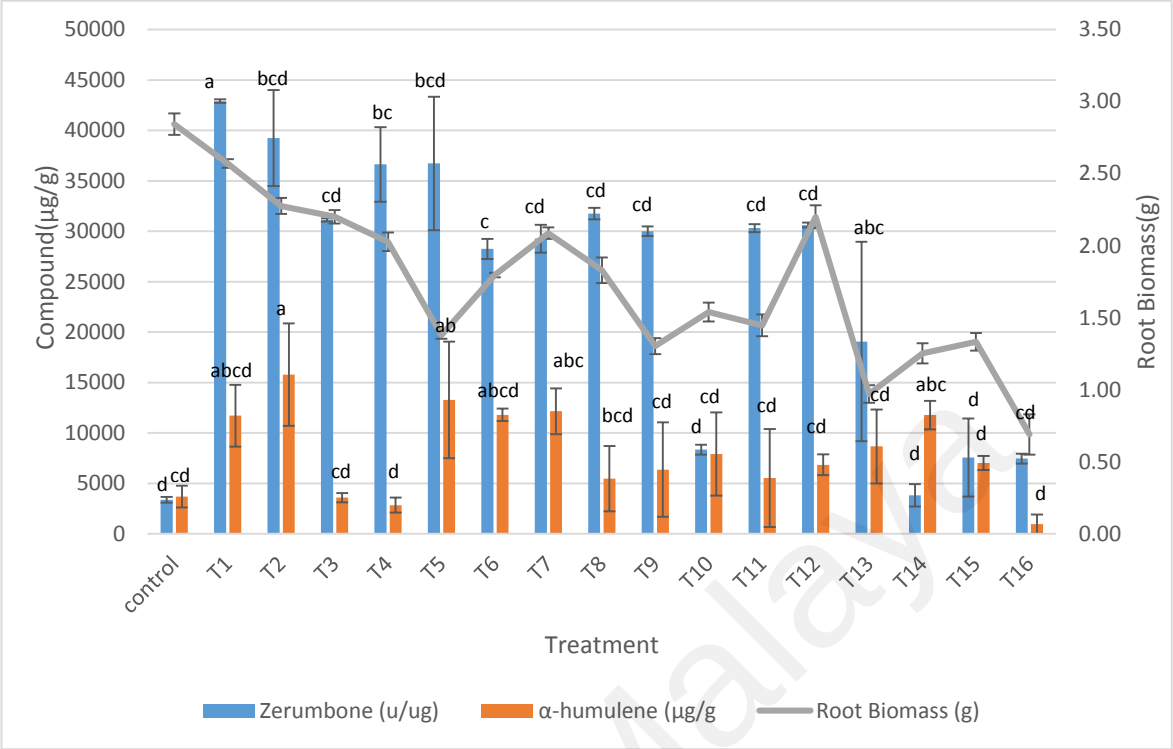

Figure 4.13: Synergistic effects of MeJA and SA on zerumbone, α-humulene and root biomass of *Z. zerumbet* after 26 days of culture prior elicitation. *T1*: MS media supplemented with 400 µM MeJA + 400 µM SA; *T2*: MS media supplemented with 400 µM MeJA + 600 µM SA; *T3*: MS media supplemented with 400 µM MeJA + 800 µM SA; *T4*: MS media supplemented with 400 µM MeJA + 1200 µM SA; *T5*: MS media supplemented with 600 µM MeJA + 400 µM SA; *T6*: MS media supplemented with 600 µM MeJA + 600 µM SA; *T7*: MS media supplemented with 600 µM MeJA + 800 µM SA; *T8*: MS media supplemented with 600 µM MeJA + 1200 µM SA; *T9*: MS media supplemented with 800 µM MeJA + 400 µM SA; *T10*: MS media supplemented with 800 µM MeJA + 600 µM SA; *T11*: MS media supplemented with 800 µM MeJA + 800 µM SA; *T12*: MS media supplemented with 800 µM MeJA and 1200 µM SA; *T13*: MS media supplemented with 1200 µM MeJA + 400 µM SA; *T14*: MS media supplemented with 1200 µM MeJA + 600 µM SA; *T15:* MS media supplemented with 1200 µM MeJA + 800 µM SA; *T16*: MS media supplemented with 1200 µM MeJA + 1200 µM SA. Error bar indicates standard deviation of the mean value. Means with different letters in the same column are significantly different at $p < 0.05$ according to ANOVA and Tukey's multiple range test.

Table 4.5: Synergistic effects of different concentration combinations of Methyl Jasmonate and Salicyclic Acid on zerumbone, α-humulene and root biomass production of *Z. zerumbet* with elicitation point at day 15 after 26 days of cultivation following elicitation.

Methyl Jasmonate (µM)	Salicylic Acid (µM)	Zerumbone (× 10^3 µg/g)	α-humulene (× 10^3 µg/g)	Root Biomass (g)
control		3.4 ± 0.3^{d}	3.7 ± 1.1^{cd}	2.8 ± 0.1^{a}
400	400	43.0 ± 0.2^{a}	11.7 ± 3.1^{abcd}	2.4 ± 0.1^{b}
400	600	39.3 ± 4.8^{bcd}	15.8 ± 5.1^{a}	2.3 ± 0.1^{bc}
400	800	31.1 ± 0.2^{cd}	3.6 ± 0.5^{cd}	2.2 ± 0.1^{bcd}
400	1200	36.6 ± 6.6^{bc}	2.9 ± 0.8^{d}	2.0 ± 0.1^{cd}
600	400	36.7 ± 0.7^{bcd}	13.3 ± 0.6^{ab}	1.4 ± 0.1^{hg}
600	600	28.2 ± 1.0^{c}	11.8 ± 0.6^{abcd}	1.8 ± 0.1^{e}
600	800	29.3 ± 0.1^{cd}	12.2 ± 2.3^{abc}	2.1 ± 0.1^{bc}
600	1200	31.2 ± 0.6^{cd}	5.5 ± 3.2^{bcd}	1.8 ± 0.1^{de}
800	400	30.0 ± 0.5^{cd}	6.4 ± 4.7^{cd}	1.3 ± 0.1^{f}
800	600	8.4 ± 0.5^{d}	7.9 ± 4.1^{cd}	1.5 ± 0.1^{f}
800	800	30.3 ± 0.4^{cd}	5.5 ± 4.8^{cd}	1.5 ± 0.1^{hg}
800	1200	31.0 ± 0.2^{cd}	6.9 ± 1.0^{cd}	2.2 ± 0.1^{bcd}
1200	400	19.1 ± 9.9^{d}	8.7 ± 7.7^{cd}	1.0 ± 0.1^{g}
1200	600	3.8 ± 1.1^{d}	11.8 ± 1.4^{abcd}	1.2 ± 0.1^{f}
1200	800	7.6 ± 3.9^{d}	17.1 ± 7.0^{a}	1.3 ± 0.1^{f}
1200	1200	7.5 ± 0.5^{d}	8.6 ± 1.0^{cd}	0.7 ± 0.1^{h}

Means with different letters in the same column are significantly different at $p < 0.05$ according to ANOVA and Tukey's multiple range test

4.5 Larger volume production in bioreactor and determination of root biomass growth and secondary metabolite production in adventitious root cultures of *Z. zerumbet*

Larger volume cultivation of adventitious root cultures was carried out in a bioreactor (Figure 4.17). Root cultures aged one month old with initial inoculum density of 10 g FW were cultured in a 5-L balloon type bubble column bioreactor (BTBCB) supplied with 1.0 L/min filtered air at 25 ± 1 °C. The working volume of the cultivation was at 1-L

using optimized media obtained from shake flask system (media supplemented with 1 mgL^{-1} NAA and 3 mgL^{-1} BAP at 16:08 light regime); pH of the liquid medium was kept constant at pH 5. Based on Figure 4.16, root biomass harvested after 30 days of culture generated 44.3 ± 4.0 g FW and 8.5 ± 1.0 g DW. The zerumbone and α-humulene produced were at 3.7 ± 1.3 x (10^3 µg/g) and 11.3 ± 2.2 x (10^3 µg/g), respectively.

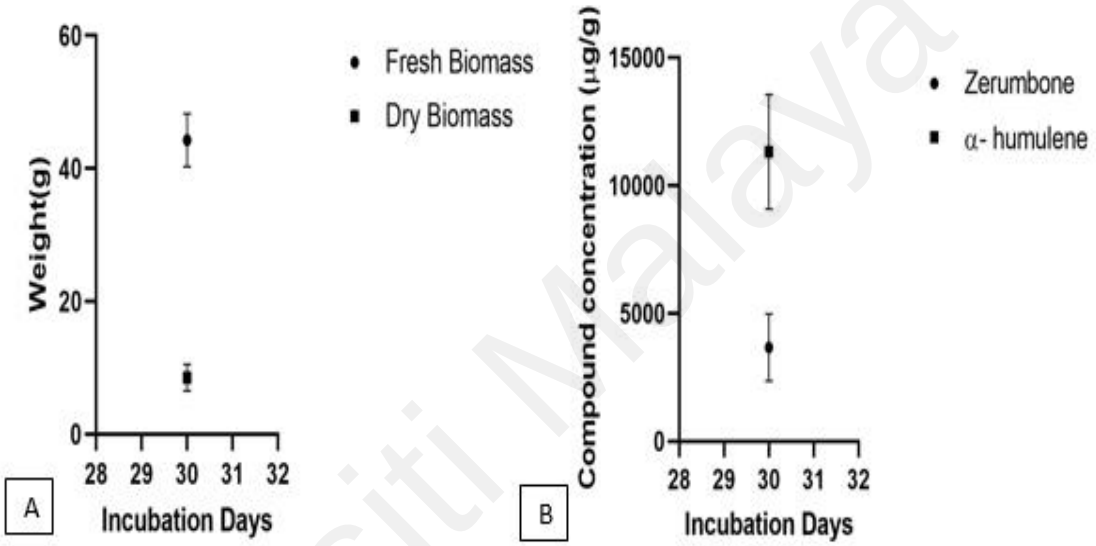

Figure 4.14: Target compounds and root biomass produced in BTBCB *A*: Fresh and dry biomass of root harvested; *B*: Zerumbone and α-humulene accumulation in harvested root.

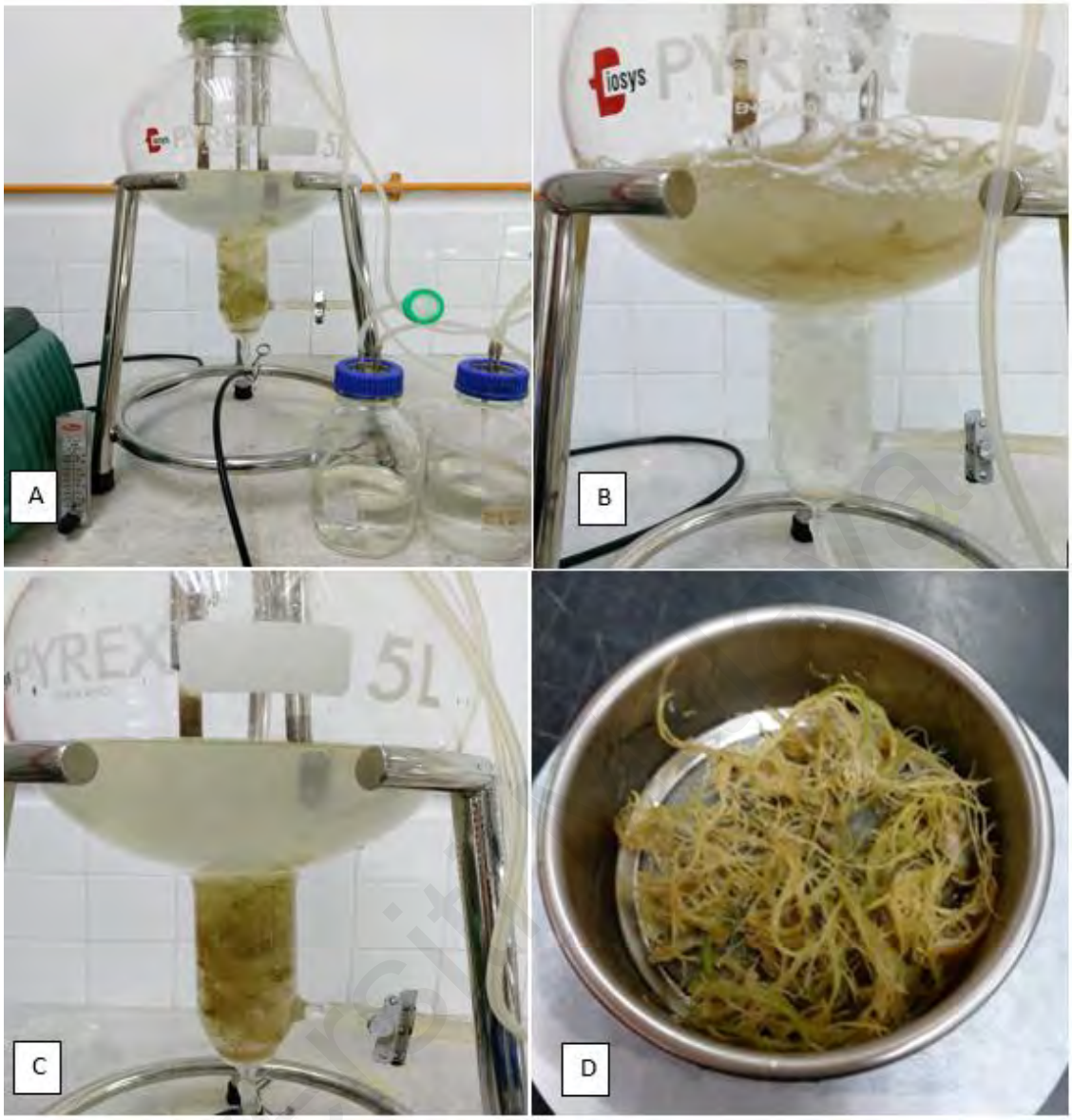

Figure 4.15: Larger volume cultivation of adventitious root culture of *Z. zerumbet* in BTBCB *A*: Roots cultivated in BTBCB at day 1 *B*: Root growth at day 15 with 1.0 L/min air flow rate *C*: Adventitious root culture after 30 days cultivation *D*: Roots harvested from BTBCB and washed with distilled water.

CHAPTER 5: DISCUSSION

5.1 Establishment of adventitious root culture of *Z. zerumbet*

5.1.1 Initiation of adventitious root cultures

Adventitious root is seen as a viable route for secondary metabolite production due to its high proliferation rate, fast growth and high potential for stable production of valuable secondary metabolites (Murthy *et al.,* 2008, Murthy *et al.*, 2016). The primary induction conditions such as pH, light regime and induction media are crucial to produce and maintain the highest yield of proliferated roots during *in-vitro* propagation. In this study, evaluation of optimum induction medium is based on the number of primary roots that are numerous, healthy along with fastest induction.

The adventitious roots induced in the investigation emerged as early as seven days after inoculation, and this varied with concentration and combinations of PGR supplied. Saiman *et al.* (2012) showed similar root emergence of *Gynura procumbens,* which appeared after seven days of inoculation. The other studies showed a delayed induction time such as the adventitious roots of *Aloe vera* L. *viz.* observed during six weeks of induction (Lee *et al.*, 2011), *Plumbago zeylanica* L. root cultures were induced in auxins supplemented medium within two weeks of incubation (Sivanesan & Jeong, 2009) and a transformed hairy roots were also induced in *Boerhavia difussa, Datura innoxia* and *Solanum xanthocarpum* at between 12^{th}-15^{th} day after infection with *Agrobacterium rhizogenesis* (Kathodia & Biswas, 2012).

Zhang *et al.* (2014) found that the formation of adventitious roots is strongly influenced by the type and concentration of auxins and IBA as inducers, which is in agreement with the finding in this study. Media supplemented with 1.0 mgL^{-1} NAA and 1.0 mgL^{-1} IBA produced maximum number of roots after 10 days of culture (18.0 ± 1.0) with rooting percentage of 93 %. The results agree with the observation of Saiman *et al.* (2012) where direct root induction was observed in the presence of IBA, and grew into fine, thin and long roots.

Even though media supplemented with 1.0 mgL^{-1} NAA and 1.0 mgL^{-1} IBA produced highest number of root initials (18.0 ± 1.0), the optimum medium with highest root responses (100 %), fastest root growth (seven days) and longest root length with high degree of branching was obtained in media supplemented with 1.0 mgL^{-1} NAA and 2.0 mgL^{-1} IBA under dark condition (Table 4.1). This is in contrast to the study by Zhang *et al.* (2013) that found the ratio of PGR for other species is different e.g. the highest root production of *Psammosilene tunicoides* was obtained in low ratio of IBA to NAA.

Exogenous supplementation of certain auxins can stimulate the differentiation and induction processes in rooting. High cytokinin-to-auxin usually trigger shoot regeneration while a high ratio of auxin-to-cytokinin will induces root regeneration, respectively. The rhizogenic action of auxins also has been deemed most crucial for the initiation and development of adventitious roots, and the physiological stages of rooting coincide with variations in endogenous and exogenous auxin concentrations. From the results, the frequency of root response, number of roots per explant, root length, zerumbone and α-humulene production were affected by the concentration and types and concentrations of auxins used for adventitious regeneration and multiplication.

There are several other reports on optimized adventitious root induction using different combination and concentration of PGR such as *Plumbago rosea* in 1.5 mgL^{-1} IAA and 1 mgL^{-1} IBA (Silja & Satheeshkumar, 2015), and roots of *Aloe vera* in 0.5 mgL^{-1} NAA and 0.2 mgL^{-1} BAP (Lee *et al.,* 2011), maximum root numbers of *Artemisia vulgaris* in MS media supplemented with 2 mgL^{-1} IAA and 1 mgL^{-1} IBA (Sujatha & Kumari, 2012), and highest root biomass of *Hypericum perforatum* L. in MS media with 0.1 mgL^{-1} kinetin and 1 mgL^{-1} IBA (Cui *et al.*, 2011).

5.1.2 Root multiplication in shake-flask system

Establishment of adventitious root culture (AdRC) in liquid medium, and establishing its growth conditions such as obtaining optimum medium components and cultivation parameters for biomass and metabolite accumulation is one of key stages as mentioned by Murthy *et al.* (2016). In this respect, the establishment of culture was done in MS media with 1 mg L^{-1} NAA supplementation and different concentrations of IBA and BAP. For treatment with auxin-auxin (NAA + IBA), there was a significant increase in adventitious root growth with increasing concentration of IBA up to 5mgL^{-1} in both light regimes. The observation is similar to Ling *et al.* (2009), where an increase in rooting efficiency of *Orthosiphon stamineus* was observed with increasing IBA and NAA concentrations from 1 mgL^{-1} to 5 mgL^{-1}, with favorable concentration for both hormones at 5 mgL^{-1}.

In this study, the maximum fresh weight (6.9 ± 0.1 g FW) and dry weight (2.1 ± 0.0 g DW) were obtained in 1 mgL^{-1} NAA supplemented with 5 mgL^{-1} IBA and 16:08 photoperiod. The results are supported by Amer *et al.* (2016) and Mona *et al.* (2019) where they found that combination of 0.5 mgL^{-1} NAA and 1.0 mgL^{-1} IBA enhanced the

root growth of *Cichorium endivia* but in dark condition. Meanwhile, Hadizadeh *et al.* (2016) also reported that *Cichorium intybus* L. roots growth is increased when subcultured into half-strength MS liquid medium supplemented with 0.2 mgL^{-1} NAA and 0.5 mgL^{-1} IBA, under continuous agitation at 110 rpm in total dark condition. Zhang *et al.* (2013) also indicated that the combination of NAA and IBA significantly affect the adventitious roots formation in *Psammosilene tunicoides* culture.

In this study, auxin-cytokinin (NAA + BAP) treatment was also investigated in order to enhance the root growth. Naseem *et al.* (2015) suggested that new hormonal control should be introduced after the root establishment in which auxin should be at lower concentration for root meristem maintenance and cytokinin supplied for root tissue differentiation. This was corroborated by a good yield of roots produced (6.8 ± 0.1 g FW) at 1 mgL^{-1} NAA supplemented with 3 mgL^{-1} BAP in 16:08 photoperiod compared to auxin-auxin treatment. Moreover, Lee *et al.* (2011) also observed a gradual increase in root dry weight of *Aloe vera* in MS media supplemented with NAA (0.5 mgL^{-1}) along with BAP (0.2 mgL^{-1}).

However, in this study poor multiplication of adventitious roots was observed at the highest concentration of IBA (7 mg L^{-1}) as high concentration of auxin and cytokinin could be stressful to the cell and inhibit root growth. Jalil *et al.* (2018) stated that inhibition of adventitious roots in *Z. zerumbet* culture could due to herbicidal properties of auxins at high concentration with regards to direct and indirect pathway of root formation. In addition, excess auxin in medium will force the plant to produce other hormones to balance it thus slowing down cell elongation and root growth leading to root apical dormancy (Chao *et al.,* 2006, Ling *et al.,* 2009).

Ling *et al.* (2009) demonstrated that further increase of auxin to 7 mgL^{-1} of IBA and NAA caused a decrease in rooting ability of *Orthosiphon stamineus,* similar to observations by Syros *et al.* (2004) and Rout *et al.* (2000) for inhibition in rooting of *Psoralea corylifolia* and *Ebenus cretica,* respectively, when the concentration of auxins are higher than the optimum. Contrary to the study by Lulu *et al.* (2015), *Eurycoma longifolia* root culture was not inhibited in medium supplemented with 7.0 or 9.0 mgL^{-1} NAA. This could be due to other factors such as species variation that gives separate response to the same type of plant growth regulator (Wei *et al.,* 2006) and the concentration of auxin resulting in either stimulated or inhibited root production (Overvoorde *et al.*, 2010).

Adventitious root production is a complex process that involves numerous endogenous plant growth regulators as well as environmental stimuli such as light and wounding. However, there was no significant difference was observed in both light regime (16:08 photoperiod and dark) in root multiplication process. Nevertheless, the experiment on the effect of light regime were suitable to be done in the root induction process.

5.2 Establishment of HPLC detection parameters for zerumbone and α-humulene

The establishment of solvent system and elution gradient to elute zerumbone and α-humulene compound was carried out to optimize resolution and accomplish good peak profiles. Adjustment to concentration for every standard compound was also made to improve resolution results. Standard calibration consisted of both compounds were developed at λ= 200 nm, 243 nm and 254 nm. The three wavelengths were chosen from

earlier literature showing maximum absorption of α-humulene at 200 to 210 nm (Musenga *et al.*, 2006) while zerumbone at 254 nm (Jalil *et al.*, 2015).

Many compounds are UV-detectable at above 240 nm wavelength (Komae & Hayashi, 1975, Baser *et al*., 2015) such as monoterpenes, diterpenes and sesquisterpernes (Strack *et al.,* 1980, Nour *et al.,* 2013). This study showed that α-humulene could be strongly detected at λ= 200 nm but giving weak signal at λ= 254 nm. Conversely, zerumbone gave strong detection signal at λ= 254 nm but weaker at λ= 200 nm. Nonetheless, both compounds showed best absorption at λ =243 nm. It is also shown in this study the retention times for α-humulene and zerumbone are at 5.7-7.5 and 9.5- 12.1 minutes respectively.

5.3 Synergistic effects of plant growth regulator (PGR) on root biomass growth and targeted secondary metabolite production

5.3.1 Combined effects of auxin-auxin (NAA and IBA)

Improvement of root growth was carried out to produce a sustainable amount of root biomass and compounds for a larger volume production. Since auxins are required in promoting growth, rooting and callus proliferation, the effects of combination of auxins towards biomass growth and compound accumulation were investigated. The highest amount of zerumbone (2984 ± 61 µg/g) was obtained in MS supplemented with 1mgL^{-1} NAA and 1 mgL^{-1} IBA; the highest α-humulene titre (3659 ± 356 µg/g) was obtained in MS supplemented 1 mgL^{-1} NAA and 5 mgL^{-1} IBA propagated at 16:08 photoperiod (Figure 4.11). The highest root biomass amount (2.1 ± 0.01 g) was observed in media MS supplemented with 1 mgL^{-1} NAA and 5 mgL^{-1} IBA at 16:08 photoperiod but

producing signifficantly lower amount of zerumbone (325 ± 34 µg/g) and a relatively higher accumulation of α-humulene (3576 ± 388 µg/g). Jalil *et al.* (2018) also found that root biomass of *Z. zerumbet* was produced at highest rate although low in zerumbone production when cultivated in MS supplemented with 1 mgL^{-1} NAA under dark condition. Lulu *et al.* (2015) found higher concentrations of IBA and NAA ranging from 5 mgL^{-1} to 9 mgL^{-1} enhanced the accumulation of total phenolics and flavonoids in *Eurycoma longifolia* contrary to the current study where low zerumbone (137 ± 30 ug/g) and α-humulene (384 ± 205 µg/g) productions were observed in MS supplemented with 1 mgL^{-1} NAA and 7 mgL^{-1} IBA at 16:08 photoperiod with low root biomass i.e. 0.6 ± 0.03 g DW.

Light is known to influence growth organogenesis, formation of plant products and enzyme induction (Zhu *et al.*, 2015). In the auxin-auxin treatment, the amounts of α-humulene and zerumbone were significantly higher at 16:08 photoperiod than dark condition. Similar observation was made by Zhu *et al.* (2015) where vindoline and sepertine biosynthesis in cultured cells, leaves, seedlings and plants of *Catharanthus roseus* increased significantly following light exposure treatment. Total anthraquinone, flavonoids and phenolic contents were found to be significantly higher in *Morinda citrifolia* root cultures when grown in MS media supplemented with 1 mgL^{-1} IBA under far-red light exposure (Baque *et al.,* 2010).

5.3.2 Combined effects of auxin-cytokinin (NAA and BAP)

Molecular mechanism regulated by auxin and cytokinin occurs during root elongation process. The effects of the two PGR on root production and secondary metabolite accumulation were studied. The amounts of zerumbone (3440 ± 168 µg/g) and α-

humulene (3759 ± 798 μg/g) were highest in MS media supplemented with 1 mgL^{-1} NAA and 3 mgL^{-1} BAP at 16:08 photoperiod with 1.90 ± 0.05 g (DW) of root biomass (Figure 4.13) compared to auxin-auxin treatment. Similar to Zhu *et al.* (2015), they reported that in *Catharanthus roseus* root culture, auxins down-regulate the expression of genes associated with terpenoid indole alkaloid biosynthesis while cytokinin acts as accelerant that enhances the accumulation of alkaloids. In addition, investigation by Tank and Thaker (2014) showed that BAP yielded a positive response in re-initiation of vegetative growth of roots that undergo endo-reduplication. Maximum plumbagin production was observed in *Plumbago rosea* when cultivated in media containing auxin and cytokinin (Silja and Satheeshkumar, 2015).

Although plant growth regulators could be applied to enhance growth of adventitious root, it could inhibit the accumulation of compounds. In this study, significant decrease in root biomass growth and compound production was observed with increased concentration of BAP. The lowest amounts of zerumbone (137 ± 7 μg/g) and α-humulene (60 ± 22 μg/g) were obtained in media containing highest BAP treatment (7 mgL^{-1}), which also produced lowest root biomass 0.4 ± 0.02 g (DW). Previous study of *Z. zerumbet* by Jalil et *al.* (2018) showed higher zerumbone production was obtained with increased auxin concentration in single treatment of PGR while highly suppressed the biomass accumulation. This could due to the herbicidal property of PGR at high concentration that inhibit the root formation and consequently lowered compound accumulation. Thus, to ensure sustainable growth and compound yield, roots were maintained in the optimum medium (MS medium supplemented with 1 mgL^{-1} NAA and 3 mgL^{-1} BAP) for further elicitation experiments and larger volume metabolite production.

5.4 Synergistic effects of Methyl Jasmonate (MeJA) and Salicylic acid (SA) as elicitors on root growth and secondary metabolite production

To enhance the production of zerumbone and α-humulene with minimal adverse effect on the root growth in the adventitious root cultures of *Z. zerumbet*, roots were grown in the combination of MeJA + SA concentrations. Figure 4.15 shows quantity of root biomass, zerumbone and α-humulene produced in the MeJA + SA elicited root cultures after 26 days following elicitation. Root biomass growth decreased with the increase in MeJA and SA concentration. Generally, when the roots were treated with MeJA and SA, root biomass growth decreased by 35.6 % under treatment T5 (600 µM MeJA + 400 µM SA) relative to control; treatment T15 (1200 µM MeJA + 800 µM SA) showed decrease in root biomass by 66.0 % relative to control.

Reduction in root biomass in following these treatments could be due to inhibitory effects by the elicitors. The inhibitory effect of MeJA on plant growth was reported by Cosio *et al.* (1990) and Jalil *et al.* (2018), where the accumulation of biomass may be hindered by the addition of elicitors when primary metabolism responsible for the growth of cells switches to the secondary metabolism following elicitation. Lois *et al.* (1989) and Sukito & Tachibana (2016) reported that concentrations above 0.01 mM SA inhibit root growth in some species meanwhile treatment with 5 mM SA showed 25 % reduction in *Vicia faba* L. root growth. Zhang *et al.* (2000) also observed similar effect of inhibition in *Taxus sp.* cell cultures.

Treatment with highest concentration of elicitors (1200 µM MeJA + 1200 µM SA) yielded lowest amount of root biomass (0.69 g) which is 75.7 % decrease compared to

control after 26 days following elicitation. The results corroborated with the single treatment of MeJA and SA undertaken by Jalil *et al.* (2018).

The combination of MeJA and SA showed less inhibition on root and compound production when treated at lower combination. Although root biomass decreased after treatment with elicitors, no significant difference was observed for combined elicitors with concentration ranging from 400 µM to 800 µM compared to control. Xu *et al.* (2015) suggested that 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 content in *Vitis vinifera* cell suspension cultures. In this study, the amount of zerumbone was higher than α-humulene in all treated root cultures of *Z. zerumbet* as shown in Figure 4.15. It is suggested that combination of MeJA and SA stimulated the conversion of α-humulene thus assisted in the synthesis of zerumbone.

The combined effects of MeJA and SA resulted in the highest production of zerumbone and α-humulene in adventitious root cultures of *Z. zerumbet*. Similar to Sukito and Tachibana (2016) whom found that synergism effects of MeJA and SA at 0.1 mM increased bilobalide and ginkgolide production in *Ginkgo biloba* cultures. From this study, treatment with 400 µM MeJA + 400 µM SA resulted in highest improvement of zerumbone production (43,000 ± 200 µg/g) representing 13-fold increase, whereas treatment with 400 µM MeJA + 600 µM SA produced highest α-humulene (15,800 ± 5100 µg/g) representing 4.3-fold increase after elicitation compared to control. This can be explained by the changes in metabolomics pathways thus increased production of some metabolites (Zhu *et al.*, 2015).

MeJA is a biotic elicitor that induces signal transduction pathway involved in the biosynthesis of various secondary metabolites (Almagro *et al.*, 2009). On the other hand, SA is a compound that initiates fast defence responses against some stress factors by producing specific compounds and proteins (Li *et al.*, 2004). The synergistic effects of elicitors were found to be an ideal strategy for increasing *in vitro* metabolite production. While adding elicitor caused strong inhibition of root growth according to Murthy *et al.* (2008) and Paek *et al.* (2009), the root biomass growth in the current study was not significantly inhibited at lower concentrations of combined elicitors. Therefore, the synergism of MeJA and SA at 400 µM concentration with elicitation point of day 15 constitutes optimal condition for sustainable *in vitro* production of targeted secondary metabolites.

5.5 Root biomass growth and secondary metabolite production of adventitious roots in larger volume controlled bioreactor

Controlled bioreactor application can offer many advantages in increasing plant secondary metabolites production, and most likely to be more efficient compared to the conventional shaken culture system. It allows for stimulation of the growth rate of the culture with active aeration and controlled temperature, pH, concentration of oxygen, carbon dioxide and nutrient in the liquid medium.

There are various studies of adventitious root cultures in large volume bioreactor (500^{-1}000 L) for plant species such as *Morinda citrifolia*, *Echinacea purpura, Echinacea augustifolia* and *Hypericum perforatum* (Baque *et al.,* 2012). Paek *et al.* (2009) reported the establishment of adventitious root culture system for the production of gingneosides from *Panax ginseng* in controlled bioreactor. Recent work by Lulu *et al.* (2015) enhanced

root biomass growth and bioactive compounds production in adventitious root culture of *Eurycoma longifolia* using larger volume controlled bioreactor system.

In this study, balloon type bubble column bioreactor (BTBCB) as designed by Paek *et al.* (2005) was used instead of column bioreactor to prevent excessive foaming problem as mentioned by Baque *et al.* (2012) and Wendy *et al.* (2014). Root cultures aged one month-old with initial inoculum density of 10 g FW were cultivated in a 5-L BTBCB at 25 ± 1°C.

Adequate aeration is necessary inside the bioreactor so as to favour good cell growth and accumulation of secondary metabolites. Relatively high aeration rate at 1.0 L/min improved the supply of filtered air to cells in the bioreactor culture, enabling cells at the center of their roots to receive adequate diffused oxygen. Jalil *et al.* (2018) also found that highest specific growth rate was achieved at 10 g inoculum density while no significant difference was observed in zerumbone accumulation in *Z. zerumbet* adventitious root culture compared to other inoculum densities. On the other hand, Shohael *et al.* (2014) suggested that higher inoculum densities (7 and 9 gL^{-1}) caused reduction in accumulated biomass of *Eleutherococcus senticosus* cultures and its eleutherosides production.

The pH of the liquid medium was kept constant at pH 5.7 within a working volume of 1 liter. The roots were cultivated in optimized medium formulation obtained from shake flask system. Root biomass was harvested after 30 days of culture with three-fold biomass increment from initial, and 3.7 ± 1.3 ($\times 10^3$ μg/g) and 11.3 ± 2.2 ($\times 10^3$ μg/g) of zerumbone and α-humulene were obtained, respectively.

Thus, improvements in implementing key factors e.g. air flow rate, inoculum density, pH, culture medium are important in enhancing root growth and compound production for bioreactor cultivation. The improved cultivation conditions for root growth as shown in this study, applied in conjunction with the elicitation strategy could be implemented to achieve high zerumbone and α-humulene production in a controlled bioreactor system.

CHAPTER 6: CONCLUSION

In this study, enhancement of α-humulene and zerumbone production in adventitious root cultures of *Z. zerumbet* Smith through synergistic application of elicitors are investigated. Improved biomass production via two-stage culture system is achieved by first cultivating the cells in induction medium supplemented with 1.0 mgL^{-1} NAA and 2.0 mgL^{-1} IBA under dark condition, whereby highest root responses (100 %), fastest root growth (seven days) and longest root length with high degree of branching are obtained. Subsequently, successful establishment of adventitious root culture (AdRC) is obtained in MS media supplemented with 1 mg L^{-1} NAA and different concentrations of IBA and BAP.

Despite the maximum yield of fresh weight (6.9 ± 0.1 g FW) and dry weight (2.1 ± 0.0 g DW) obtained in MS media supplemented with 1 mgL^{-1} NAA and 5 mgL^{-1} IBA under 16:08 photoperiod, zerumbone accumulation is significantly low (325 ± 34 µg/g). On the other hand, production of zerumbone (3440 ± 168 µg/g) and α-humulene (3759 ± 798 µg/g) are highest in MS media supplemented with 1 mgL^{-1} NAA and 3 mgL^{-1} BAP under 16:08 photoperiod from 1.90 ± 0.05 g (DW) of root biomass. Thus, adventitious root culture in this formulation is selected for further elicitation study.

Adventitious root cultures of *Z. zerumbet* are elicited using the combination of Methyl jasmonate (MeJA) and Salicylic acid (SA) to enhance the production of zerumbone and α-humulene with minimal adverse effects on the root growth. The combination of elicitors show relatively less inhibition of root and good compound enhancement effects when tested at a concentration between range of 400 µM to 800 µM. The amount of zerumbone is consistently higher than α-humulene in all treated root cultures of *Z. zerumbet.* It is proposed that the mixture of MeJA and SA induces significant intracellular

conversion of α -humulene, thereby leading to enhanced biosynthesis of zerumbone. Treatment at day 15 of cultivation with 400 μM MeJA + 400 μM SA results in 13-fold increase in zerumbone yield (43,000 ± 200 μg/g), while treatment with 400 μM MeJA + 600 μM SA give highest increase at 4.3-fold in α-humulene yield (15,800 ± 5100 μg/g) compared to control.

Scaling up of adventitious root cultures of *Z. zerumbet* in 5-L Balloon Type Bubble Column Bioreactor (BTBCB) is also investigated. By applying the improved medium formulation obtained earlier and under cultivation parameters of 25 ± 1 ° C, controlled pH 5.7, aeration rate of 1.0 vvm, root culture aged one month inoculum of 10 g FW, a three-fold increase in biomass after 30 days of cultivation, and 3,700 ± 1300 μg/g and 11,300 ± 2200 μg/g of zerumbone and α-humulene are achieved, respectively.

For future studies, it is recommended to investigate the nature of elicitation by these compounds through metabolomics analysis where the information obtained may be applied to further improve the yields of α-humulene and zerumbone.

REFERENCES

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

Achan,, J., Talisuna, A. O., Erhart, A., Yeka, A., Tibenderana, J. K., Baliraine, F. N., Rosenthal, P. J., & D'Alessandro, U. (2011). Quinine, an old anti-malarial drug in a modern world: role in the treatment of malaria. *Malaria Journal*, *10*, 144.

Ahmad J., Imran K., Renald B., Joseph A., Mohamad F. M. (2020) *Stevia rebaudiana* Bertoni: an updated review of its health benefits, industrial applications and safety. *Trends in Food Science & Technology, 100*, 177-189.

Ahmad S., Hahn E.J., Paek Y. K. (2008) Aeration volume and photosynthetic photon flux affect cell growth and secondary metabolite contents in bioreactor cultures of *Morinda citrifolia*. *Journal Plant Biology*, 51,209–212.

Ahmad, N., Fatima, N., Bullet, I., Ahmad, I., & Anis, M. (2015) Effect of PGRs in adventitious root culture in vitro: present scenario and future prospects. *Rendiconti Lincei*. 26, 307-321.

Ahmed, S. A., & Baig, M. M. V. (2014). Biotic elicitor enhanced production of psoralen in suspension cultures of *Psoralea corylifolia* L. *Saudi Journal of Biological Sciences*, *21*, 499-504.

Aka Kacar, Y., Biçen, B., Şimşek, Ö, Dönmez, D., & Erol, M. (2020). Evaluation and Comparison of a New Type of Temporary Immersion System (Tis) Bioreactors for Myrtle (*Myrtus Communis* L.). *Applied Ecology and Environmental Research. 18*. 1611-1620.

Akhtar, N., Jantan, I., Arshad, L., & Haque, M. A. (2019). Standardized ethanol extract, essential oil and zerumbone of *Zingiber zerumbet* rhizome suppress phagocytic activity of human neutrophils. *BMC complementary and alternative medicine*, *19*(1), Article #331.

Ali, M., Abbasi, B. H., Ali, G. S. (2015) Elicitation of antioxidant secondary metabolites with jasmonates and gibberellic acid in cell suspension cultures of *Artemisia absinthium* L. *Plant Cell Tissue Organ Culture, 120*(3), 1099–1106.

Almagro, L., Go´mez Ros, L., Belchi-Navarro, S., Bru R., RosBarcelo´ A., & Pedren˜o M. (2009) Class III peroxidases in plant defence reactions. *Journal of Experimental Botany,60,377*–390.

Alutbi, S.D., Al-Saadi, S.A., & Madhi, Z.J. (2017). The Effect of Salicylic Acid on the Growth and Microtuberization of Potato (*Solanum tuberosum* L.) cv. Arizona Propagated *in-vitro*. *Journal of Biology, Agriculture and Healthcare, 7*, 64-70.

Amasino, R. (2005). 1955: Kinetin arrives. The 50th anniversary of a new plant hormone. *Plant Physiology, 138*, 1177–1184.

Anaya, F., Fghire, R., Wahbi, S., & Loutfi, K. (2017). Antioxidant enzymes and physiological traits of *Vicia faba* L. as affected by salicylic acid under salt stress. *Journal of Materials and Environmental Science, 8*(7), 2549-2563.

Archana, C. P., Geetha S. P., & Balachandran I. (2013). *In vitro* microrhizome induction in three high yielding cultivars of *Zingiber officinale* Rosc. and their phytopathological analysis. *International Journal of Advance Biotechnology and Research,* 4(2), 296-300.

Arif T. (2015). Salicylic acid as a peeling agent: a comprehensive review. *Clinical, cosmetic and investigational dermatology*, 8, 455–461.

Arigundam U., Asokan M. V., Yaw L. S., Dawn M. & Samir C. D. (2020). Liquid culture for efficient in vitro propagation of adventitious shoots in wild *Vaccinium vitis-idaea* ssp. minus (lingonberry) using temporary immersion and stationary bioreactors. *Scientia Horticulturae, 264* (5), 1091-1099.

Ashraf, S. M., Sebastian, J., & Rathinasamy, K. (2019). Zerumbone, a cyclic sesquiterpene, exerts antimitotic activity in HeLa cells through tubulin binding and exhibits synergistic activity with vinblastine and paclitaxel. *Cell proliferation*, *52*(2), Article #12558.

Azhar, S. Z. A., Ghani, K. A., & Yusuf, N. A., 2018. *In Vitro* Induction of Adventitious Root from Shoot Bud of *Boesenbergia rotunda* (Zingiberaceae): Effect of Plant Growth Regulators. *Science International (Lahore), 30*(1), 147-151.

Badshah, S. L., Ullah, A., Ahmad, N., Almarhoon, Z. M., & Mabkhot, Y. (2018). Increasing the Strength and Production of Artemisinin and Its Derivatives. *Molecules 23*(1), 100.

Balakrishnan, R. K., Radzan, R. K., & Bhattacharyya, S. C. (1956). Oil of *Zingiber zerumbet* Smith. Part 1: Isolation of various constituents and characterization of the inonocyclic ketone zerumbone. *Perfumery Essential Oil Record*, 47,274–279.

Baque A., Murthy, H.N., & Paek, K.Y. (2014). Adventitious Root Culture of Morinda citrifolia in Bioreactors for Production of Bioactive Compounds", in K. Y. Paek, N. M. Hosakatte, J. J. Zhong (Eds.), *Production of Biomass and Bioactive Compounds Using Bioreactor Technology* (pp. 185-22). Springer Science.

Baque, M. A., Elgirban, A., Lee, E. J., & Paek, K. Y. (2011). Sucrose regulated enhanced induction of anthraquinone, phenolics, flavonoids biosynthesis and activities of antioxidant enzymes in adventitious root suspension cultures of *Morinda citrifolia* (L.). *Acta Physiology Plant*, *34*, 405-415.

Baque, M. A., Hahn, E. J., Paek, K. Y., (2010). Induction mechanism of adventitious root from leaf explants of *Morinda citrifolia* as affected by auxin and light quality. *In Vitro Cell Development Biology Plant,* 46, 71-80.

Baque, M. A., Moh, S. H., Lee, E. J., Zhong, J. J., & Paek, K. Y. (2012). Production of biomass and useful compounds from adventitious roots of high-value added medicinal plants using bioreactor. *Biotechnology Advances, 30*(6), 1255–1267.

Baque, M. A., Shiragi, M. H. K., Moh, S. H., Lee, E. J., & Paek, K. Y. (2013). Production of biomass and bioactive compounds by adventitious root suspension cultures of *Morinda citrifolia* (L.) in a liquid-phase airlift balloon-type bioreactor. *In Vitro Cellular & Developmental Biology in Plant*, 49, 737-749.

Bartholomew, D.P. (2014). History and perspectives on the role of ethylene in pineapple flowering. *Acta Horticulturae, 1042*, 269-284.

Baser, K. Husnu Can, & Buchbauer, G. (2015). *Handbook of Essential Oils: Science, Technology, and Applications,* (2nd Ed.). New York: CRC Press.

Bernal-Vicente, A., Petri, C., Hernández, J.A. & Diaz-Vivancos, P. (2020), Biochemical study of the effect of stress conditions on the mandelonitrile-associated salicylic acid biosynthesis in peach. *Plant Biology Journal, 22*, 277-286.

Bienaimé, C., Melin, A., Bensaddek, L., Attoumbré, J., Nava-Saucedo, E., & Baltora-Rosset, S. (2015). Effects of plant growth regulators on cell growth and alkaloids production by cell cultures of *Lycopodiella inundata*. *Plant Cell, Tissue and Organ Culture (PCTOC), 123*(3), 523–533.

Borthakur, M., Hazarika J., & Singh. R. S. (1998) A Protocol for Micropopagation of *Alpinia galangal*. *Plant Cell Tissue Organ Culture, 55*, 231-233.

Cao, L, Wu, H, & Zhang, H. (2020). Highly efficient production of diverse rare ginsenosides using combinatorial biotechnology. *Biotechnology and Bioengineering*, *117*, 1615– 1627.

Chadwick, M., Trewin, H., Gawthrop, F., & Wagstaff, C. (2013). Sesquiterpenoids lactones: benefits to plants and people. *International Journal of Molecular Sciences*, *14*(6), 12780–12805.

Chamovitz, D. (2020). *What A Plant Knows* (pp. 29-30). United States of America: Scientific American.

Chandrasekaran, S., Veronica, J., Sundar, S., & Maurya, R. (2017). Alcoholic fractions F5 and F6 from *Withania somnifera* leaves show a potent antileishmanial and immunomodulatory activities to control experimental visceral leishmaniasis. *Frontiers in Medicine*, *12*(4), 55.

Chandrasekhar, B., Umesha, S., & Naveen, K. H. N. (2017). Proteomic analysis of salicylic acid enhanced disease resistance in bacterial wilt affected chilli (*Capsicum annuum*) crop. *Physiology Molecular Plant Pathology,* 98, 85–96.

Chane-Ming, J., Vera, R., & Chalchat, J. C. (2003). Chemical composition of the essential oil from rhizomes, leaves and flowers of *Zingiber zerumbet* Smith from Reunion Island. *Journal of Essential Oil Research, 15*(3), 202-205.

Chin, W. Y. W., Annuar, M. S. M., Tan, B. C., & Khalid, N. (2014). Evaluation of a laboratory scale conventional shake flask and a bioreactor on cell growth and regeneration of banana cell suspension cultures. *Scientia Horticulture, 172*, 39–46.

Christoffersson, J., & Mandenius, C. F. (2019). Using a microfluidic device for culture and drug toxicity testing of 3D cells. *Methods in Molecular Biology*, 235–241.

Cosio, E. G., Frey, T., & Verduyn, R. (1990). High-affinity binding of a synthetic heptaglucoside and fungal glucan phytoalexin elicitors to soybean membranes. *FEBS Letters,*271,223–226.

Costa, J. M., Heuvelink, E., & Van De Pol, P. (2017). Propagation by Cuttings. In *Reference Module in Life Sciences.* Elsevier.

Cui, H. Y., Murthy, H. N., Moh, S. H., Cui, Y. Y., Lee, E. J., & Paek, K. Y. (2014). Production of biomass and bioactive compounds in protocorm cultures of *Dendrobium candidum* Wall ex Lindl. Using balloon type bubble bioreactors. *Industrial Crop Production*, *53*, 28-33.

Cui, X. H., Chakrabarty, D., Lee, E. J., & Paek, K. Y. (2010). Production of adventitious roots and secondary metabolites by *Hypericum perforatum* L. in a bioreactor. *Bioresource Technology*, 101, 4708-4716.

Cui, X., Murthy, H.N. & Zhang, J., (2020). Effect of nutritional factors on the accretion of secondary metabolites in Malaysian ginseng adventitious root cultures. *Plant Biotechnology Reports, 14*, 381–386.

Cutler, S. R., Rodriguez, P. L., Finkelstein, R. R., & Abrams, S. R. (2010). Abscisic acid: emergence of a core signaling network. *Annual review of plant biology, 61*, 651–679.

Da Silva, J.A., Solis-Gracia, N. & Jifon, J. (2020). Use of bioreactors for large-scale multiplication of sugarcane (*Saccharum spp*.), energy cane (*Saccharum spp.),* and related species. *In Vitro Cellular Developmental Biology in Plant 56*, 366–376.

Dai, J. R., Cardellina, J.H., Mc Mohan, J.B., & Boyd M.R. (1997). Zerumbone, an HIV-inhibiotory cytotoxic sesquiterpene of *Zingiber aromaticum* and *Zingiber zerumbet*. *Natural Product Letters, 10*(2), 115-118.

Dar T. A., Uddin M., Khan M. M. A., Hakeem K. R., Jaleel H. (2015). Jasmonates counter plant stress: a review. *Environmental and Experimental Botany, 115*, 49–57.

Dash, S., Dey, P., Kundu, A., Kumar, A., Gupta, M., Lee, B. M., Bhakta, T., & Kim, H. S. (2020). Analysis of alkaloids (indole alkaloids, isoquinoline alkaloids, tropane alkaloids). *Recent Advances in Natural Products Analysis*, 505–567.

Davies, P. J. (2010). The plant hormones: their nature, occurrence, and function. In Davies P. J. (Eds.), *Plant hormones: biosynthesis, signal transduction, action*, (pp. 1-15). Springer- Dordrecht.

Deepthi, S., & Satheeshkumar, K. (2017). Cell line selection combined with jasmonic acid elicitation enhance camptothecin production in cell suspension cultures of *Ophiorrhiza mungos* L. *Applied Microbiology and Biotechnology, 101*(2), 545–558.

Dev, S. (1960). Studies in sesquiterpenes-XVI. Zerumbone, a monocyclic sesquiterpene ketone. *Tetrahedron*, *8*(3&4), 171–180.

Dicosmo, F., & Misawa, M. (1995). Plant cell and tissue culture: alternatives for metabolite production. *Biotechnology Advances, 13*, 425-453.

Diwan, R., & Malpathak, N. (2011). Bioprocess optimization of furanocoumarin elicitation by medium renewal and re-elicitation: a perfusion-based approach. *Applied Biochemistry and Biotechnology*, *163*(6), 756–764.

Donnez, D., Kim, K. H., Antoine, S., Conreux, A., De Luca, V., Jeandet, P., & Clément, C. (2011). Bioproduction of resveratrol and viniferins by an elicited grapevine cell culture in a 2 L stirred bioreactor. *Process Biochemistry, 46*, 1056–1062.

Durango, D., Quiñones, W., Torres, F., Rosero, Y., Gil, J., & Echeverri F. (2002). Phytoalexin accumulation in Colombian bean varieties and aminosugars as elicitors. *Molecules*, *7*, 817 - 832.

Endang, R., & Youngmin K. (2020) Yeast metabolic engineering for the production of pharmaceutically important secondary metabolites. *Applied Microbiology in Biotechnology, 104*, Article #4659.

Enders, T. A., & Strader, L. C. (2015). Auxin activity: Past, present, and future. *American Journal of Botany, 102*(2), 180–196.

Etemad, L., Moshiri, M., Balali-Mood, M. (2019) Advances in treatment of acute sulfur mustard poisoning – a critical review, *Critical Reviews in Toxicology, 49*(3), 191-214.

Faizal, A., & Sari, A. V. (2019). Enhancement of saponin accumulation in adventitious root culture of Javanese ginseng (*Talinum paniculatum* Gaertn.) through methyl jasmonate and salicylic acid elicitation. *African Journal of Biotechnology, 18*(6), 130-135.

Faridah, Q. Z, Abdelmageed, A. H. A., Julia, A. A., & Nor H., R. (2011). Efficient *in vitro* regeneration of *Zingiber zerumbet* Smith (a valuable medicinal plant) plantlets from rhizome bud explants. *African Journal of Biotechnology*, *10*(46), 9303-9308.

Ferri, M., Dipalo, S. C. F., Bagni, N., & Tassoni, A. (2011). Chitosan elicits mono-glucosylated stilbene production and release in fed-batch bioreactor cultures of grape cells. *Food Chemistry, 124*, 1473–1479.

Filho, C., Souza, B. A., Leila. T., Braz, R. J., & Tavares M. (2000). Curcuma: medicinal, spice and of other potential use plant. *Ciencia Rural*, 30, 171-175.

Finkelstein, R. (2013). Abscisic Acid synthesis and response. *The Arabidopsis Book*, *11*, Article #0166.

Fujita, Y., Hara, Y., Suga, C., & Morimoto T. (1981). Production of shikonin derivative by cell suspension culture of *Lithospermum erythrorhizon. Plant Cell Reports, 1*, 61-63.

Gadzovska, S., Maury, S., Delaunay, A., Spasenoski, M., Hagège, Courtois, D., & Joseph, C. (2013). The influence of salicylic acid elicitation of shoots, callus, and cell suspension cultures on production of naphtodianthrones and phenylpropanoids in *Hypericum perforatum* L. *Plant Cell Tissue and Organ Culture*, *113*(1), 25-39.

Gao, Y., Honzatko, R. B., & Peters, R. J. (2012). Terpenoid synthase structures: a so far incomplete view of complex catalysis. *Natural Product Reports, 29*(10), 1153–1175.

Gaosheng, H. & Jingming, J. (2012) Production of useful secondary metabolites through regulation of biosynthetic pathway in cell and tissue suspension culture of medicinal plants. *Recent Advances in Plant in vitro Culture*. London, UK. pp 11:197.

Garcia, C., Furtado de Almeida, A., & Costa, M. (2019). Abnormalities in somatic embryogenesis caused by 2, 4-D: an overview. *Plant Cell Tissue Organ Culture*, *137,* 193–212.

George, E., Hall, M., & Klerk, G. J. (2008). The components of plant tissue culture media. II. Organic additions, osmotic and pH effects and support systems. In George, E., Hall, M., & Klerk, G. J. (Eds), *Plant Propagation by Tissue Culture* (pp. 115-173). Netherland: Springer.

Georgiev, M., Ludwig-Müller, J., Weber, J., Stancheva, N., & Bley, T. (2010). Bioactive metabolite production and stress-related hormones in Devil's claw cell suspension cultures grown in bioreactors. *Applied Microbiology and Biotechnology, 89*, 1683–1691.

Germana, M. A. (2011). Anther culture for haploid and doubled haploid production. *Plant Cell, Tissue and Organ Culture, 104*, 283-300.

Gerth, A., Schmidt, D. & Wilken, D. (2007). The production of plant secondary metabolites using bioreactors. *Acta Horticulturae*, *764*, 95–104.

Ghazalee, S., Haque, M. A., Jantan, I., & Harikrishnan, H. (2019). Standardized extract of *Zingiber zerumbet* suppresses LPS-induced pro-inflammatory responses through NF-κB, MAPK and PI3K-Akt signaling pathways in U937 macrophages. *Phytomedicine: International Journal of Phytotherapy and Phytopharmacology*, *54*, 195–205.

Ghorpade R. P., Chopra A., Nikam T. D. (2011) Influence of biotic and abiotic elicitors on four major isomers of boswellic acid in callus culture of *Boswellia serrata* Roxb. *Plant Omics Journal*, *4*, 169–176.

Giri, C.C., & Zaheer, M. (2016). Chemical elicitors versus secondary metabolite production in vitro using plant cell, tissue and organ cultures: recent trends and a sky eye view appraisal. *Plant Cell, Tissue and Organ Culture (PCTOC), 126*, 1-18.

Girisa, S., Shabnam, B., Monisha, J., Fan, L., Halim, C. E., Arfuso, F., Ahn, K. S., Sethi, G., & Kunnumakkara, A. B. (2019). Potential of Zerumbone as an Anti-Cancer Agent. *Molecules*, *24*(4), Article #734.

Grzegorczyk, I., & Wysoki ´ nska, H. (2010). Antioxidant compounds in *Salvia officinalis* L. shoot and hairy root cultures in the nutrient sprinkle bioreactor. *Acta. Societatis Botanicorum Poloniae, 79,* 7–10.

Hadizadeh, H., Mohebodini, M., & Esmaeilpoor, B. (2016). Effects of auxins on induction and establishment of adventitious and hairy roots culture of the medicinal plant chicory (*Cichorium intybus* L.). *Iran Journal of Medical Aromatic Plants*. *32*, 389–396.

Hamirah M. N., Sani H. B., Boyce P.C., & Sim S. L. (2010). Micropropagation of red ginger (*Zingiber montanum* Koenig), a medicinal plant. *Asia-Pacific Journal of Molecular Biology and Biotechnology, 18*, 127-30.

Hao, Y.J., An, X. L., Sun H. D., Piao X. C., Gao R. & Lian M. L., (2020). Ginsenoside synthesis of adventitious roots in *Panax ginseng* is promoted by fungal suspension homogenate of *Alternaria panax* and regulated by several signaling molecules. *Industrial Crops and Products, 150,* Article # 112414.

Hedden, P., & Sponsel, V. (2015). A Century of Gibberellin Research. *Journal of Plant Growth Regulation*, *34*(4), 740–760.

Hezari, M., Ketchum, R. E., Gibson, D. M. & Croteau, R. (1997). Taxol production and taxadiene synthase activity in *Taxus canadensis* cell suspension cultures. *Archives of Biochemistry and Biophysics, 337*(2), 185-190.

Hohm, T., Preuten, T. & Fankhauser, C. (2013). Phototropism: Translating light into directional growth. *American Journal of Botany*, *100* (1): 47–59.

Hrahsel, L., & Thangjam, R. (2013). Strategies for large-scale production of commercially important banana varieties of Mizoram, India, using plant tissue culture technique. *Science Vission*, *13*(3), 137-141.

Huang, G. C., Chien, T. Y., Chen, L. G., & Wang, C. C. (2005). Antitumor effects of zerumbone from *Zingiber zerumbet* in P-388D cells *in vitro* and *in vivo*. *Planta Medica*, *71(*3), 219-224.

Husen, A., Iqbal, M., & Sohrab, S.S. (2018). Salicylic acid alleviates salinity-caused damage to foliar functions, plant growth and antioxidant system in Ethiopian mustard (*Brassica carinata* A. Br.). *Agriculture & Food Security*, *7*, Article #44.

Husin, N., Jalil, M., Othman, R. Y., & Khalid N. (2014). Enhancement of regeneration efficiency in banana (*Musa acuminata* cv. Berangan) by using proline and glutamine. *Scientia Horticulture, 168*, 33-37.

Hussain, G., Rasul, A., Anwar, H., Aziz, N., Razzaq, A., Wei, W., Ali, M., Li, J., & Li, X. (2018). Role of Plant Derived Alkaloids and Their Mechanism in Neurodegenerative Disorders. *International Journal of Biological Sciences*, *14*(3), 341–357.

Hussein R.A., & El-Anssary A.A. (2019) Plants secondary metabolites: the key drivers of the pharmacological actions of medicinal plants. In Philip F. Builders (Eds.).

Ibrahim, M. M., El-Bahr, M. & Rady, M. (2019). In-vitro adventitious root production of *Cichorium endivia* L. and antioxidants, total phenolic, and total flavonoids assessments.*Egyptian Pharmaceutical Journal*, *18*, 216-227.

Idris F.D., Jalil M., Ibrahim H., Khalid N. (2009) Establishment of embryogenic cell suspension and field evaluation of re-generants of *Zingiber zerumbet* Smith. *Acta Horticulturae*, *829* (6).

Imaneh, D., Akbar, M., & Gholamreza, A. (2011). *In vitro* and *in vivo* Production of Gingerols and Zingiberene in Ginger Plant (*Zingiber officinale* Roscoe). *Iranian Journal of Pharmaceutical Sciences*, *7* (2), 117-121.

Iqbal, N., Khan, N., Ferrante, A., Trivellini, A., Francini, A., & Khan, M. (2017). Ethylene Role in Plant Growth, Development and Senescence: Interaction with Other Phytohormones. *Frontiers in Plant Science*, *8,* 475.

Ivanov, I, Berkov, S, Pavlov, A, & Georgiev, V. (2019). In sito galanthamine extraction during the cultivation of *Leucojum aestivum* L. shoot culture in two-phase bubble column cultivation system. *English Life Science,* 19, 1000– 1005.

Jalil, M. (2018). Enhancement and Isolation of Zerumbone in *Zingiber Zerumbet* (L.) Smith Cell Suspension and Adventitious Root Cultures. Doctoral dissertation. Retrieved from Universiti Malaya. Kuala Lumpur. Malaysia.

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*, 1-7.

Jalili-Nik, M., Sadeghi, M. M., Mohtashami, E., Mollazadeh, H., Afshari, A. R., & Sahebkar, A. (2020). Zerumbone Promotes Cytotoxicity in Human Malignant Glioblastoma Cells through Reactive Oxygen Species (ROS) Generation. *Oxidative Medicine and Cellular Longevity*, Article #3237983.

Jamal, A., Javed, K., Aslam, M. & Jafri, M. A. (2006). Gastroprotective effect of cardamom, *Elettaria cardamomum* Maton. Fruits in rats. *Journal of Ethnopharmacology, 103*, 149–153.

Jang, H. I., Rhee, K. J., & Eom, Y. B. (2020). Antibacterial and antibiofilm effects of α-humulene against *Bacteroides fragilis*. *Canadian Journal of Microbiology*, *66*(6), 389–399.

Jang, H., Lee, H., & Shohael, A.M. (2016). Production of biomass and bioactive compounds from shoot cultures of *Rosa rugosa* using a bioreactor culture system. *Horticulture Environmental Biotechnology, 57***,** 79–87.

Jantan, I., Haque, M. A., Ilangkovan, M., & Arshad, L. (2019). Zerumbone from *Zingiber zerumbet* inhibits innate and adaptive immune responses in Balb/C mice. *International Immunopharmacology*, *73*, 552–559.

Jeandet, P., Clément, C., Tisserant, L. P., Crouzet, J. & Courot, E. (2016). Use of grapevine cell cultures for the production of phytostilbenes of cosmetic interest. *Comptes Rendus Chimie*, 19(9), 1062-1070.

Jendoubi, W., Harbaoui, K. & Hamada, W. (2017). Salicylic acid-induced resistance against *Fusarium oxysporum*.s.pradicis lycopercisi in hydroponic grown tomato plants. *Journal of New Science Agriculture Biotechnology, 21*, 985–995.

Jenifer, U., Francina, C. K., & Ravindhran, R. (2012). *In vitro* adventitious root and hairy root cultures in *Boerhaavia diffusa* L. *International Journal of Current Research*, *4*(1), 65-67.

Kannan N., Manokari M., & Shekhawat S. M. (2020). Enhanced production of anthraquinones and phenolic compounds using chitosan from the adventitious roots of *Morinda coreia. Industrial Crops and Products, 148*, Article #112321.

Kayalvizhi, T., Thiyagarajan, M., & Venkatachalam, P. (2013). Micropropagation of *Alpinia officinarum*: an anti–cancer medicinal plant. *International Journal Plant Cell Biotechnology of Molecular Biology. 14*(1&2), 60–65.

Khan, N. A., Mir, M. R., Nazar, R., & Singh, S. (2008). The application of ethephon (an ethylene releaser) increases growth, photosynthesis and nitrogen accumulation in mustard (*Brassica juncea* L.) under high nitrogen levels. *Plant Biology. 10*, 534–538.

Khanam, M. N., & Anis, M. (2018). Organogenesis and efficient in vitro plantlet regeneration from nodal segments of *Allamanda cathartica* L. using TDZ and ultrasound assisted extraction of quercetin. *Plant Cell Tiss Organ Culture, 134*, Article #241.

Khatodia, S., & Biswas, K. (2012). A Comparative Study of Hairy Roots Induction in Medicinally Important Plants by *Agrobacterium rhizogenes* MTCC 532. *International Journal of Current Microbiology and Applied Science, 3*(5), 625-633.

Kieber, J. J. (2002). Tribute to Folke Skoog: Recent Advances in our Understanding of Cytokinin Biology. *Journal of Plant Growth Regulator, 21* (1), 1–2.

Kieber, J. J., & Schaller, G. E. (2014). Cytokinins. *The Arabidopsis Book*, *12*, Article #0168.

Kim, J. W., Yang, D., & Jeong, H. (2019). Dietary zerumbone, a sesquiterpene, ameliorates hepatotoxin-mediated acute and chronic liver injury in mice. *Phytotherapy Research*, *33,* 1538– 1550.

Kim, Y. S., Hahn, E. J., Murthy, H. N., Paek, K. Y. (2004). Adventitious root growth and ginsenoside accumulation in *Panax ginseng* cultures as affected by methyl jasmonate. *Biotechnology Letter, 26*, 1619–1622.

Klessig, D. F., Choi, H. W., & Dempsey, D. M. A. (2018). Systemic acquired resistance and salicylic acid: past, present, and future. *Mol. Plant-Microbe Interaction,* 31, 871–888.

Koga, A. Y., Beltrame, F. L., & Pereira, A. V. (2016). Several aspects of *Zingiber zerumbet*: a review. *Brazillian Journal of Pharmacognosy*, *26*, 385-391.

Komae, H., & Hayashi, N. (1975). Separation of essential oils by liquid chromatography. *Journal of Chromatography. 114*, 258-260.

Krishnamurti, C., & Rao, S. C. (2016). The isolation of morphine by Serturner. *Indian Journal of Anaesthesia, 60*(11), 861–862.

Kusakari,K., Yokoyama, M., Inomata, S. & Gozu,Y. (2012). Large scale production of saiko saponins through root culturing of *Bupleurum falcatum* L. using modified airlift reactors. *Journal of Bioscience and Bioengineering, 113*, 99–105.

Labban L. (2014). Medicinal and pharmacological properties of turmeric (*Curcuma longa*): A review. *Pharmacology Biomedical Scince Journal, 5*(1), 17-23.

Largia, M. J. V., Pothiraj, G., Shilpha, J., & Ramesh, M. (2015). Methyl jasmonate and salicylic acid synergism enhances bacoside A content in shoot cultures of *Bacopa monnieri* (L.). *Plant Cell Tissue Organ Culture, 122*(1), 9-20.

Lee, E., Jiménez, Z., Seo, K., Nam, G.B., Kang, Y., Lee, T.R., Kim, D., & Yang, D. (2019). Mass production of coumestrol from soybean (*Glycine max*) adventitious roots through bioreactor: effect on collagen production. *Plant Biotechnology Reports, 14*, 110 - 99.

Lee, E.-J., & Paek, K. Y. (2012) Enhanced productivity of biomass and bioactive compounds through bioreactor cultures of *Eleutherococcus koreanum* Nakai adventitious roots affected by medium salt strength. *Industrial Crop Products, 36*, 460–465.

Lee, E.-J., Park, S.-Y., & Paek, K. Y. (2015). Enhancement strategies of bioactive compound production in adventitious root cultures of *Eleutherococcus koreanum* Nakai subjected to methyl jasmonate and salicylic acid elicitation through airlift bioreactors. *Plant Cell Tissue Organ Culture, 120*, 1–10.

Lee, I. C., Hong, S. W., Whang, S. S., Lim, P. O., Nam, H. G., & Koo, J. C. (2011). Age-dependent action of an ABA-inducible receptor kinase, RPK1, as a positive regulator of senescence in Arabidopsis leaves. *Plant Cell Physiology. 52*, 651–662.

Lee, K. J., Park, Y., & Kim, J. Y. (2015) Production of biomass and bio-active compounds from adventitious root cultures of *Polygonum multiflorum* using air-lift bioreactors. *Journal of Plant Biotechnology, 2*(1), 34–42.

Lee, Y. S., Yang, T. J., Park, S. U., Baek, J. H. Wu, S. (2011). Induction and proliferation of adventitious roots from *Aleo vera* leaf tissues for *in vitro* production of aloe-emodin. *Plant Omics Journal*., *4*, 190-194.

Li, P., Zhu, N., Hu, M., Wu, H., Yu, T., Wu, T., Zhang, D., Sun, Z., Yang, J., Ma, G., & Xu, X. (2017). New cucurbitane triterpenoids with cytotoxic activities from Hemsleya penxianensis. *Fitoterapia*, *120*, 158–163.

Li, Z., Bai, Y., Huang, C., & Cai, Y. (2016). Novel delay-partitioning stabilization approach for networked control system via Wirtinger-based inequalities. *ISA Transactions, 61*, 75–86.

Ling, A. P. K., Kok, K. M., & Hussein S. (2009). Adventitious rooting of *Orthosiphon stamineus* in response to sucrose concentrations and medium pH. *American Eurasian Journal of Sustainable Agriculture 3,* 93–10.

Lois, R., Dietrich, A., Hahlbrock, K., & Schulz, W. (1989). A phenylalanine ammonia-lyase gene from parsley: structure, regulation and identification of elicitor and light responsive *cis*-acting elements. *The EMBO Journal, 8*, 1641–1648.

Lott, A. A., Baklajian, E. R., Dickinson, C. C., Collakova, E., & Jelesko, J. G. (2019). Accession-Level Differentiation of Urushiol Levels, and Identification of Cardanols in Nascent Emerged Poison Ivy Seedlings. *Molecules*, *24*(23), Article #4213.

Loyola-Vargas, V. M., & Vázquez-Flota, F. (2006). An introduction to plant cell culture: Back to the future. *Methods in molecular biology, 318*, 3–8.

Lu, J. J., Bao, J. L., Chen, X. P., Huang, M., & Wang, Y. T. (2012). Alkaloids isolated from natural herbs as the anticancer agents. *Evidence-based complementary and alternative medicine: eCAM*, Article #485042.

Lulu, T., Park, S. Y., Ibrahim, R., & Paek, K. Y. (2015). Production of biomass and bioactive compounds from adventitious roots by optimization of culturing conditions of *Eurycoma longifolia* in balloon-type bubble bioreactor system. *Journal of Bioscience and Bioengineering*, *119*(6), 712-717.

Mahdieh, M., Noori, M., & Hoseinkhani, S. (2015). Establishment of In vitro Adventitious Root Cultures and Analysis of Flavonoids in *Rumex crispus. Plant Tissue Culture and Biotechnology*, *25*(1), 63-70.

Malik, S., Cusido, R., Mirjalili, M., Moyano, E., & Palazon, J. (2011). Production of the anticancer drug taxol in *Taxus baccata* suspension cultures: A review. *Process Biochemistry. 46.* 23-34.

Manokari, M., & Shekhawat, M. S. (2016). Implications of auxins in induction of adventitious roots from leaf explants of cannon ball tree (*Couroupita guianensis* Aubl.) *World Scientific News*, *33*,109-121.

Maruri-López, I., Aviles-Baltazar, N. Y., Buchala, A., & Serrano, M. (2019). Intra and Extracellular Journey of the Phytohormone Salicylic Acid. *Frontiers in plant science*, *10*, 423.

Mendoza, D., Cuaspud, O., Arias, J. P., Ruiz, O., & Arias, M. (2018). Effect of salicylic acid and methyl jasmonate in the production of phenolic compounds in plant cell suspension cultures of *Thevetia peruviana. Biotechnology Reports*, *19*, Article #00273.

Michel, P., Granica, S., Magiera, A., Rosińska, K., Jurek, M., Poraj, Ł., & Olszewska, M. A. (2019). Salicylate and Procyanidin-Rich Stem Extracts of *Gaultheria procumbens* L. Inhibit Pro-Inflammatory Enzymes and Suppress Pro-Inflammatory

and Pro-Oxidant Functions of Human Neutrophils *Ex-Vivo*. *International journal of Molecular Sciences*, *20*(7), Article #1753.

Miller, C. O., Skoog, F., von Saltza, M. H. & Strong, F. M. (1955). "Kinetin, a cell division factor from deoxyribonucleic acid". *Journal of the American Chemical Society, 77,* Article #1392.

Mona, M. I., Mohamed, E. B., Rady, K, & Mohamed R. (2019). *In-vitro* adventitious root production of *Cichorium endivia* L. and antioxidants, total phenolic, and total flavonoids assessments. *Egyptian Pharmaceutical Journal, 18*(3), 216-227.

Murashige, T., & Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiology Plant*, *15*, 473-479.

Murthy H. N., Dandin V. S. & Paek K. Y. (2016) Tools for biotechnological production of useful phytochemicals from adventitious root cultures. *Phytochemistry Reviews, 15,*129–145.

Murthy, H. N., Dandin, V. S., Zhong, J. J. & Paek, K. Y. (2014). Strategies for enhanced production of plant secondary metabolites from cell and organ cultures. In: Paek K. Y., Murthy H. N., Zhong J. J., eds. *Production of Biomass and Bioactive Compounds using Bioreactor Technology*. Dordrecht: Springer, 471–508.

Murthy, H. N., Dijkstra, C., Anthony, P., White, D. A., Davey, M. R., Powers, J. B., Hahn, E. J., & Paek, K. Y. (2008). Establishment of *Withania somnifera* hairy root cultures for the production of Withanolide A. *Journal of Integrative Plant Biology*, *50*, 915-981.

Murthy, H. N., Hahn, E. J., & Paek, K. Y. (2008). Adventitious roots and secondary metabolism. *Chinese Journal of Biotechnology, 24*, 711-716.

Musenga, A., Ferrantil, A., Saracinol, M. A., Fanali, S., & Raggil, M.A. (2006). Simultaneous determination of aromatic and terpenic constituents of cloves by means of HPLC with diode array detection. *Journal of Separation Science*. *29*, 1251–1258.

Naing, A. H., Kim, S. H., Chung, M. Y., Park, S. K., & Kim, C. K. (2019). In vitro propagation method for production of morphologically and genetically stable plants of different strawberry cultivars. *Plant methods*, *15*, Article #36.

Nalawade, S. M., Sagare, A. P., Lee, C. Y., Kao, C. L., & Tsay, H. S. (2003). Studies on tissue culture of Chinese medicinal plant resources in Taiwan and their sustainable utilization. *Botanical Bulletin of Academia Sinica*, *44*(2), 79-98.

Nam, B., So, Y., Kim, H. Y., Kim, J. B., Jin, C. H., & Han, A. R. (2017). A New Monoterpene from the Leaves of a Radiation Mutant Cultivar of *Perilla frutescens* var. crispa with Inhibitory Activity on LPS-Induced NO Production. *Molecules, 22*(9), Article #1471.

Naseem, M., Kaltdorf, M., & Dandekar T. (2015). The nexus between growth and defence signalling: auxin and cytokinin modulate plant immune response pathways, *Journal of Experimental Botany*, *66* (16), 4885–4896.

Nivelle, L., Hubert, J., Courot, E., Borie, N., Renault, J., H., Nuzillard, J. M., Harakat, D., Clément, C., Martiny, L., & Delmas, D. (2017). Cytotoxicity of labruscol, a new resveratrol dimer produced by grapevine cell suspensions, on human skin melanoma cancer cell line HT-144. *Molecules, 22*, Article #1940.

Ogawara,, T., Higashi, K., Kamada, H., & Ezura, H. (2003). Ethylene advances the transition from vegetative growth to flowering in *Arabidopsis thaliana*. *Journal of Plant Physiology*, *160*(11), 1335–1340.

Osugi, A., & Sakakibara, H. (2015). Q&A: how do plants respond to cytokinins and what is their importance. *BMC Biology, 13*, Article #102.

Othman, H. H., Samad, N. A., Abdul, A. B., Rahman, H. S., Abdullah, R., & Tengku Ibrahim, T. A. (2019). Antiproliferative and antiangiogenic effects of zerumbone from *Zingiber zerumbet* L. Smith in sprague dawley rat model of hepatocellular carcinoma. *Pharmacognosy Magazine*. *15*, 61, 277-282.

Othman, R., Abdurasid, M., Mahmad, N., Fadzillah N. A. (2019). Alkaline-based curcumin extraction from selected zingiberaceae for antimicrobial and antioxidant activities. *Pigment & Resin Technology*, *48*(1), 293-300.

Overvoorde, P., Fukaki, H., & Beeckman, T. (2010). Auxin control of root development. *Cold Spring Harb Perspective Biology*, *2*, Article #001537.

Ozaki Y., Kawahara N., & Harada M. (1991) Anti-inflammatory effect of *Zingiber cassumunar* Roxb and its active principles. *Chemical Pharmacology Bulletin*, *39*, 2353–2356.

Paek, K. Y., Murthy, H. N., Hahn, E. J., & Zhong, J. J. (2009). Large scale culture of ginseng adventitious roots for production of ginsenosides. *Advances Biochemical Engineering Biotechnology*, *113*, 151-176.

Parthasarathy, A., Cross, P. J., Dobson, R., Adams, L. E., Savka, M. A., & Hudson, A. O. (2018). A Three-Ring Circus: Metabolism of the Three Proteogenic Aromatic Amino Acids and Their Role in the Health of Plants and Animals. *Frontiers in Molecular Biosciences*, *5*, Article #29.

Parthasarathy, A., Cross, P., Dobson, R., Adams, L., Savka, M., Hudson, A. (2018). A Three-Ring Circus: Metabolism of the Three Proteogenic Aromatic Amino Acids and Their Role in the Health of Plants and Animals. *Frontiers in Molecular Biosciences, 5*, Article #29.

Pasternak, T., Groot, E. P., Kazantsev, F. V., Teale, W., Omelyanchuk, N., Kovrizhnykh, V., Palme, K., & Mironova, V. V. (2019). Salicylic Acid Affects Root Meristem Patterning via Auxin Distribution in a Concentration-Dependent Manner. *Plant Physiology, 180*(3), 1725–1739.

Patel, N., Mohd-Radzman, N.A., Corcilius, L., Crossett, B., Connolly, A., Cordwell, S.J., Ivanovici, A., Taylor, K., Williams, J., Binos, S., Mariani, M., Payne, R.J. & Djordjevic, M.A. (2018). Diverse peptide hormones affecting root growth identified

in the Medicago truncatula secreted peptidome. *Molecular & Cellular Proteomics, 17* (1), 160-174.

Pérez-Hernández, J., Nicasio-Torres, M., Sarmiento-López, L. G., & Rodríguez-Monroy, M. (2019). Production of anti-inflammatory compounds in *Sphaeralcea angustifolia* cell suspension cultivated in stirred tank bioreactor. *English Life Science*, *19,* 196– 205.

Piao, X., Zhang, W., & Jiang, J. (2017). Cell suspension culture of *Orostachys cartilaginous* in bioreactor systems for bioactive compound production and evaluation of their antioxidant properties. *Acta Physiology Plant, 39*, Article #70.

Pierik, R., Tholen, D., Poorter, H., Visser, E. J., & Voesenek, L. A. C. J. (2006). The Janus face of ethylene: growth inhibition and stimulation. *Trends in Plant Science, 11*, 176–183.

Pirasaci T., Manisali A. Y., Dogaris I., Philippidis G. & Sunol A. K. (2017). Hydrodynamic design of an enclosed Horizontal BioReactor (HBR) for algae cultivation. *Algal Research, 28*, 57–65.

Pojer, E., Maivi, F., Johnson, D. & Stockley C. S. (2013). The case for anthocyanin consumption to promote human health: A review. *Comprehensive Revissions Food Sci Food Safety, 12* (5), 483-508.

Popova, E., Shukla, M. & Kim, H. (2020). Root cryobanking: an important tool in plant cryopreservation. *Plant Cell Tiss Organ Culture, 144*, 49–66.

Priyadarshani, S.V.G.N., Hu, B., Li, W. (2018). Simple protoplast isolation system for gene expression and protein interaction studies in pineapple (*Ananas comosus* L.). *Plant Methods 14,* Article #95.

Ptak, A., Morańska, E., Skrzypek, E., Warchoł, M., Spina, R., Laurain-Mattar, D., & Simlat, M. (2020). Carbohydrates stimulated Amaryllidaceae alkaloids biosynthesis in *Leucojum aestivum* L. plants cultured in RITA® bioreactor. *Peer Journal, 8*, Article #8688.

Punyarani K. & Sharma G. J. (2010). Micropropagation of *Costus speciosus* (Koen.) Sm. Using Nodal Segment Culture. *Science Biology, 2*(1), 58-62.

Rademacher, W. (2015). Plant Growth Regulators: Backgrounds and Uses in Plant Production. *Journal of Plant Growth Regulation, 34*, 845-872.

Radić, Sandra. (2016). Influence of pH and plant growth regulators on secondary metabolite production and antioxidant activity of *Stevia rebaudiana* (Bert). *Periodicum Biologorum. 118,* 9-19.

Rafsanjani, M. S., Alvari, A., Mohammad, A., Abdin, M. Z., & Hejazi, M. A. (2011). *In vitro* propagation of *Cichorium intybus* L. and quantification of enhanced secondary metabolite (esculin). *Recent Patents on Biotechnology, 5*(3), 227–234.

Raghavendra T. (2002). Neuromuscular blocking drugs: discovery and development. *Journal of the Royal Society of Medicine*, *95*(7), 363–367.

Rahman, H. S., Abdullah, R., Yeap, S. K., Othman, H., Chartrand, M., Namvar, F., Abdul, A., & How, C. (2014). Biomedical Properties of a Natural Dietary Plant Metabolity, Zerumbone, in Cancer Therapy and Chemoprevention Trials. *Biomedical Research International*, Article #920742.

Raju, C. S., Varutharaju, K., Thilip, C., Aslam, A., & Shajahan, A. (2015). Rhizogenesis in Cell Suspension Culture from Mango Ginger: A Source of Isosorbide and n-Hexadecanoic Acid. *Advances in Botany*, Article #942761.

Ramakrishna, A., & Ravishankar, G. A. (2011). Influence of abiotic stress signals on secondary metabolites in plants. *Plant Signaling & Behavior*, *6*(11), 1720–1731.

Ramirez-Estrada, K., Vidal-Limon, H., Hidalgo, D., Moyana, E., Golenioswki, M., Cusidó, R. M. & Palazon, J. (2016). Elicitation, an effective strategy for the biotechnological production of bioactive high-added value compounds in plant cell factories. *Molecules*, 21(2), Article #182.

Reddy, C.V.K., Sreeramulu, D., & Raghunath, M. (2010). Antioxidant activity of fresh and dry fruits commonly consumed in India. *Food Research International, 43,* 285–288.

Rout, G. R, Samantaray, S., Das, P. (2000). *In vitro* rooting of *Psoraleacory lifolia* Linn. Peroxidase activity as a marker. *Plant Growth Regulator, 30*, 215–219.

Sah, S., Reddy, K., Li, J. (2016). Abscisic Acid and Abiotic Stress Tolerance in Crop Plants. *Frontiers in Plant Science. 7*, Article #571.

Saiman, M. Z., Mustafa, N. R., Schulte, A. E., Verpoorte, R., & Choi, Y. H. (2012). Induction, characterization, and NMR-based metabolic profiling of adventitious root cultures from leaf explants of *Gynura procumbens*. *Plant Cell Tiss Organ Culture. 109*, 465-475.

Salvo, S. A. G. D., Hirsch, C.N., Buell, C. R., Kaeppler, S. M. & Kaeppler, H. F. (2014) Whole Transcriptome Profiling of Maize during Early Somatic Embryogenesis Reveals Altered Expression of Stress Factors and Embryogenesis-Related Genes. *PloS ONE, 9*(10), Article #111407.

Sangra, A., Shahin, L. & Dhir, S. K. (2019). Long-Term Maintainable Somatic Embryogenesis System in Alfalfa (*Medicago sativa*) Using Leaf Explants. *Embryogenic Sustainability Approach. Plants, 8,* Article #278.

Sarin, R. (2005). Useful Metabolite from Plant Tissue Culture. *Biotechnology*, *4*, 79-93.

Sasaki, T., Suzaki, T., Soyano, T., Kojima, M., Sakakibara, H., & Kawaguchi, M. (2014). Shoot-derived cytokinins systemically regulate root nodulation. *Natural Communications, 5*, Article #4983.

Schramm, S., Köhler, N., & Rozhon, W. (2019). Pyrrolizidine Alkaloids: Biosynthesis, Biological Activities and Occurrence in Crop Plants. *Molecules*, *24*(3), Article #498.

Sevik, H., & Guney, K. (2013). Effects of IAA, IBA, NAA, and GA3 on rooting and morphological features of *Melissa officinalis* L. stem cuttings. *The Scientific World* Journal,1, Article # 909507.

Sezigen, S., Eyison, R., & Kenar, L. (2019). Bioanalytical verification of sulfur mustard exposure in a Syrian family. *Gulhane Medical Journal, 61*, 64-68.

Shin, D. S., & Eom, Y. B. (2019). Zerumbone inhibits *Candida albicans* biofilm formation and hyphal growth. *Canadian Journal of Microbiology*, *65*(10), 713–721.

Shohael, A. M., Murthy, H. N., & Paek, K. Y. (2014). Pilot-scale culture of somatic embryos of *Eleutherococcus senticosus* in airlift bioreactors for the production of eleutherosides. *Biotechnology Letters*, *36*(8), 1727-1733.

Silja, P. K., & Satheeshkumar, K. (2015). Establishment of adventitious root cultures from leaf explants of *Plumbago rosea* and enhanced plumbagin production through elicitation. *Industrial Crop and Production*, 76, 479-486.

Simão MJ, Collin M, Garcia RO, Mansur E, Pacheco G, Engelmann F (2018) Histological characterization of *Passiflora pohlii* Mast. root tips cryopreserved using the V-Cryo-plate technique. *Protoplasma*, 255, 741–750.

Singh, Y., Girisa, S., Banik, K., Ghosh, S., Swathi, P., Deka, M. & Padmavathi, G. (2019). Potential application of zerumbone in the prevention and therapy of chronic human diseases. *Journal of Functional Foods. 53*, 248-258.

Sitarek, P., Kowalczyk, T., Picot, L., Michalska-Hejduk, D., Bijak, M., Białas, A., Wielanek, M., ´Sliwi ´ nski,, T. & Skała, E. (2018). Growth of *Leonurus sibiricus* L. roots with over-expression of AtPAP1 transcriptional factor in closed bioreactor, production of bioactive phenolic compounds and evaluation of their biological activity. *Industrial Crop Produts*, *122*, 732–739.

Sivakumar, G., Yu, K. W., & Paek, K. Y. (2005). Production of biomass and ginsenosides from adventitious roots of *Panax ginseng* in bioreactor cultures. *Engineering in Life Science*, *5*(4), 333-342.

Sivanesan, I., & Jeong B. R. (2009). Induction and establishment of adventitious and hairy root cultures of *Plumbago zeylanica* L. *African Journal of Biotechnology*, *8*(20), 5294-5300.

Skrzypczak-Pietraszek, E., Urbańska, A., Żmudzki, P. & Pietraszek, J. (2019). Elicitation with methyl jasmonate combined with cultivation in the Plantform™ temporary immersion bioreactor highly increases the accumulation of selected centellosides and phenolics in *Centella asiatica* (L.) Urban shoot culture. *English Life Science, 19*, 931–943.

Smith, T., & Calvo, A. (2014). The mtfA Transcription Factor Gene Controls Morphogenesis, Gliotoxin Production, and Virulence in the Opportunistic Human Pathogen *Aspergillus fumigatus*. *Eukaryotic cell. 13*.

Sorin, C., Bussell, J. D., Camus, I., Ljung, K., Kowalczyk, M., Geiss, G., Mckhann, H., Garcion, C., Vaucheret, H., & Sandberg, G. (2005). Auxin and light control of adventitious rooting in Arabidopsis require argonaute1. *Plant Cell*, *17*, 1343-1359.

Stanly, C., & Kang, C. L. (2007). Micropropagation of *Curcuma zedoaria* Roscoe and *Zingiber zerumbet* Smith. *Biotechnology, 6*(4), 555-560.

Steingroewer J., Bley T., & Georgiev V. (2013) Bioprocessing of dif-ferentiated plant in vitro systems. *English Life Science, 13*(1), 26–38.

Stella de Freitas T. F., Stout M. J. & Sant'Ana J. (2019). Effects of exogenous methyl jasmonate and salicylic acid on rice resistance to *Oebalus pugnax*. *Pest Management Science*, *75,*744–752.

Strack D. Proksch P., & Gülz P. G. (1980). Reversed phase high performance liquid chromatography of essential oils. *Z. Naturforsch. Journal of Bioscience, 35*,675–81.

Su, R. N., Sujarani, M., Shalini, P., & Prabhu, N. (2019). A Review on Bioreactor Technology Assisted Plant Suspension Culture. *Asian Journal of Biotechnology and Bioresource Technology*, *5*(3), 1-13.

Sujatha, G., & Kumari, B.D. (2012). Establishment of fast-growing in vitro root culture system in *Artemisia vulgaris. International Journal of Agricultural Technology, 8*, 1779-1790.

Sukito, A., & Tachibana, S. (2016). Effect of methyl jasmonate and salycilic acid synergism on enhancement of bilobalide and ginkgolide production by immobilized cell cultures of *Ginkgo biloba*. *Bioresources and Bioprocessing, 3*, Article #24.

Sun, T.-P. (2011). The molecular mechanism and evolution of the GA-GID-DELLA signaling module in plants. *Current Biology, 21,* 338-345.

Sykłowska-Baranek, K., Rymaszewski, W., Gaweł, M. (2019). Comparison of elicitor-based effects on metabolic responses of *Taxus* × media hairy roots in perfluorodecalin-supported two-phase culture system. *Plant Cell Representative, 38*(1), 85-99.

Syros, T. D., Yupsanis, T., Zafiriadis, H., & Economou A.S. (2004) Activity and isoforms of peroxidases, lignins and anatomy, during adventitious rooting in cuttings of *Ebenus cretical*. *Journal of Plant Physiology, 161,* 69–77.

Takada Y., Murakami A., &Agarwal B.B. (2005) Zerumbone abolishes NF-kappa B and I Kappa Balpha kinase activation leading to suppression of anti-apoptotic and metastatic gene expression, upregulation of apoptosis and down regulation of invasion. *Oncogene 17,* 122–130.

Tanaka, T., Shnimizu, M. & Moriwaki, H. (2012). Cancer chemoprevention by carotenoids. *Molecules, 17,* 3202–3242.

Tank, J., & Thaker, V. (2014). Systemic Control of Cell Division and Endoreduplication by NAA and BAP by Modulating CDKs in Root Tip Cells of *Allium cepa. Biomedical Research International,* Article #453707.

Tavakoli, F., Rafieiolhossaini, M., Ravash, R., & Ebrahimi, M. (2020). Subject: UV-B radiation and low temperature promoted hypericin biosynthesis in adventitious root culture of *Hypericum perforatum*. *Plant Signaling & Behavior, 15*(7), Article #1764184

Teoh, E. S. (2016). Secondary Metabolites of Plants. *Medicinal Orchids of Asia,* 59-73.

Theanphong, O., Songsak, T. & Kirdmanee, C. (2010). Effect of plant growth regulators on microprogation of *Curcuma aeruginosa* Roxb. *Thailand Journal of Botany*, 2, 135-142.

Tian, M., Wu, X., Hong, Y., Wang, H., Deng, G., & Zhou, Y. (2020). Comparison of Chemical Composition and Bioactivities of Essential Oils from Fresh and Dry Rhizomes of *Zingiber zerumbet* (L.) Smith. *Biomedical Research International*, Article #9641284.

Tiwari, R., & Rana, C. S. (2015). Plant secondary metabolites: a review. *International Journal of Engineering Research and General Science*, 3(5), 661-670.

Tripathy, S. K., & Lenka, D., Prusti, A., Mishra, D., Swain, D. & Behera, S. (2019). Anther culture in rice: Progress and breeding perspective. *Applied Biological Research, 21*, Article #87.

Trujillo-Villanueva, K., Rubio-Piña, J., Monforte-González, M., Ramírez-Benítez, E. & Vázquez-Flota, F. (2012).The sequential exposure to jasmonate, salicylic acid and yeast extract promotes sanguinarine accumulation in *Argemone mexicana* cell cultures. *Biotechnology Letters, 34*, 379–385.

Tsuchiya, H. (2017) Anesthetic agents of plant origin: a review of phytochemi-cals with anesthetic activity. *Molecules, 22*(8), Article #1369.

Umar, M. I., Asmawi, M. Z., Sadikun, A., Altaf, R. & Iqbal, M. A. (2011). Phytochemistry and medicinal properties of *Kaempferia galangal* L. (Zingiberaceae) extracts. *African Journal of Pharmacy and Pharmacology,* 5 (14), 1638-1647.

Utaka, Y., Kashiwazaki, G., Tsuchida, N., Fukushima, M., Takahashi, I., Kawai, Y., & Kitayama, T. **(**2020). Remarkable Potential of Zerumbone to Generate a Library with Six Natural Product-like Skeletons by Natural Material-Related Diversity-Oriented Synthesis. *The Journal of Organic Chemistry*. *85*(13), 8371-8386.

Varier, N. S. (1944). Chemical examination of the rhizomes of *Zingiber zerumbet*, Smith. *Proceedings of the Indian Academy of Sciences*. *20*(5), 257–260.

Vasconsuelo, A., & Boland, R. (2007). Molecular aspects of the early stages of elicitation of secondary metabolites in plants. *Plant Science,* 172, 861-875.

Veena, G.L., Dinesh, M.R., Kumar, R.A. (2015). Axillary bud culture in papaya. *Bioinfolet, 12*, 147-149.

Veeresham, C. (2012). Natural products derived from plants as a source of drugs. *Journal of Advanced Pharmaceutical Technology & Research, 3*(4), 200–201.

Vera-Sirera, F., Gomez, M. D. and Perez-Amador, M. A. (2015). DELLA proteins, a group of GRAS transcription regulators, mediate gibberellin signaling. In Plant Transcription Factors: Evolutionary, Structural and Functional Aspects (ed. D. H. Gonzalez), pp. 313-328. San Diego: Elsevier/Academic Press.

Vijaya, Sree, N., Udayasri, P. V. V, Aswani, K. Y., Ravi, B. B., Phani, K. Y., & Vijaya V. M. (2010). Advancement in the production of secondary metabolites. *Journal of Natural Products*, 3, 112-123.

Vincent, K. A., Mathew, K. M., & Hariharan, M. (1992). Micropropagation of *Kaempferia galangal* L. a medicinal plant. *Plant Cell Tissue and Organ Culture*, *28*, 229-230.

Weathers, P. J., Arsenault, P. R., Covello, P. S., McMickle, A., Teoh, K. H., & Reed, D. W. (2011). Artemisinin production in *Artemisia annua*: studies in planta and results of a novel delivery method for treating malaria and other neglected diseases. *Phytochemistry reviews: proceedings of the Phytochemical Society of Europe, 10*(2), 173–183.

Weremczuk-Je˙, zyna, I., Kochan, E., Szymczyk, P., Lisiecki, P., Ku´zma, Ł., & Grzegorczyk-Karolak, I. (2019). The antioxidant and antimicrobial properties of phenol-rich extracts of *Dracocephalum forrestii* W. W. Smith shoot cultures grown in the nutrient sprinkle bioreactor. *Phytochemistry Letter, 30,* 254–260.

Wilson, S. A., & Roberts, S. C. (2012). Recent advances towards development and commercialization of plant cell culture processes for the synthesis of biomolecules. *Plant Biotechnology Journal*, *10*(3), 249-268.

Wu, C. H., Popova, E. V., Hahn, E. J., & Paek, K. Y. (2009). Linoleic and α-linolenic fatty acids affect biomass and secondary metabolites production and nutritive properties of *Panax ginseng* adventitious roots cultured in bioreactors. *Biochemical Engineering Journal*, *47*(1-3), 109-115.

Xu, A., Zhan, J. C., & Huang, W. D. (2015). Effects of ultraviolet C, methyl jasmonateand salicylic acid alone or in combination on stilbene biosynthesis in cell suspension cultures of *Vitis vinifera* L. cv. Cabernet Sauvignon. *Plant Cell Tissue Organ Cult*ure, *122*, 197-211.

Yan, C. & Xie, D. (2015), Jasmonate in plant defence: sentinel or double agent. *Plant Biotechnology Journal, 13*, 1233-1240.

Yang, C., Chen, M., Zeng, L., Hang, L., Liu, X., Tang, K. &Liao, Z. (2011) Improvement of Tropane alkaloid production in hairy root cultures of *Atropa belladonna* by over expressing Pmt and h6h genes. *Plant Omics Journal, 4*, 29-33.

Yob, N. J., Jofrry, S., Meor Mohd Affandi, M. M. R., Teh, L. K., Salleh, M. Z., & Zakaria, Z. A. (2011). *Zingiber zerumbet* (L.) Smith: A Review of Its Ethnomedical, Chemical and Pharmalogical Uses. *Evident-Based Complementary and Alternative Medicine,* Article #543216.

Yu, F., Okamto, S., Nakasone, K., Adachi, K., Matsuda, S., Harada, H., Misawa, N., & Utsumi, R. (2008). Molecular cloning and functional characterization of α-humulene

synthase, a possible key enzyme of zerumbone biosynthesis in shampoo ginger (Zingiber zerumbet Smith). *Planta, 227*, 1291-1299.

Yuan, J. L., Yue, J. J., Wu, X. L., & Gu, X. P. (2013). Protocol for callus induction and somatic embryogenesis in Moso Bamboo. *PloS ONE, 8*(12), Article #81954.

Yue, W., Qian-liang, M., Bing, L., Khalid, R., Cheng-Jian, Z., Ting, H. & Lu-ping, Q. (2016) Medicinal plant cell suspension cultures: pharmaceutical applications and high-yielding strategies for the desired secondary metabolites, *Critical Reviews in Biotechnology, 36(*2), 215-232.

Yusnita, Y., Jamaludin, A., & Hapsoro, D. (2018). A Combination of IBA and NAA Resulted in Better Rooting and Shoot Sprouting than Single Auxin on Malay Apple, *Syzygium malaccense* (L.) Merr., & Perry Stem Cuttings. *Agrivita. 40,* 80-90.

Yusuf N. A., Rahim, N. S. M., Azhar S. Z. A., Ghani, K. A., Sommano, S., & Khalid N. (2018) adventitious root cultures of *Boesenbergia rotunda* as a source of Pinostrobin. *International Journal on Advance Science Engineering and Information Technology, 8*, 377-383.

Yusuf, N. A., Annuar, M. S. M & Khalid, N. (2013). Existance of bioactive flavonoids in rhizomes and plant cell cultures of *Boesenbergia rotunda* (L.) Mansf. Kulturpfl. *Australian Journal of Crop Science,* 7(6), 730-734.

Yusuf, N. A., Annuar, M. S. M., & Khalid, N. (2011). Rapid micropropagation of *Boesenbergia rotunda* (L.) Mansf. Kulturpfl. (a valuable medicinal plant) from shoot bud explants. *African Journal of Biotechnology*, *10*(7), 1194-1199.

Zaheer M., Giri C. C. (2015). Multiple shoot induction and jasmonic versus salicylic acid driven elicitation for enhanced andrographolide production in Andrographis paniculata. *Plant Cell Tissue and Organ Culture, 122*(3), 553–63.

Zaheer M., Reddy V. D., & Giri C. C. (2016) Enhanced daidzin production from jasmonic and acetyl salicylic acid elicited hairy root cultures of *Psoralea corylifolia* L. (Fabaceae), *Natural Product Research, 30*(13), 1542-1547.

Zaheer, M. & Giri, C.C. (2017). Enhanced diterpene lactone (andrographolide) production from elicited adventitious root cultures of *Andrographis paniculata*. *Research on Chemical Intermediates 43***,** 2433–2444.

Zare, N., Farjaminezhad, R., Asghari-Zakaria, R., Farjaminezhad, M. (2014). Enhanced thebaine production in Papaver bracteatum cell suspension culture by combination of elicitation and precursor feeding. *Natural Product Research, 28*, 711–717.

Zhang, C., Mei, X., & Liu, L. (2000). Enhanced paclitaxel production induced by the combination of elicitors in cell suspension cultures of *Taxus chinensis*. *Biotechnology Letters, 22*, 1561–1564.

Zhang, F., Shi, J. J., Thakur, K., Hu, F., Zhang, J. G. & Wei, Z. J. (2017). Anti-Cancerous Potential of Polysaccharide Fractions Extracted from Peony Seed Dreg on Various

Human Cancer Cell Lines Via Cell Cycle Arrest and Apoptosis. *Frontiers in Pharmacology, 8*, Article #102.

Zhang, H., Han, W., De Smet, I., Talboys, P., Loya, R., & Hassan, A. (2010). ABA promotes quiescence of the quiescent center and suppresses stem cell differentiation in the Arabidopsis primary root meristem. *Plant Journal, 64*, 764–774.

Zhang, L., & Lu, S. (2017). Overview of Medicinally Important Diterpenoids Derived from Plastids. *Mini Reviews in Medicinal Chemistry*, *17*(12), 988–1001.

Zhang, X., Li, Y., Cui, L., Lei, J.M., & Li, Q. (2014). Effects of different concentrations of organic affixture on the growth and secondary metabolites contents in adventitious roots of *Tripterygium willfordii. Plant Science Journal, 32*(2), 174-180.

Zhang, Y., Mo, L., Chen, F., Lu, M., Dong, W., Wang, Q., Xu, F., & Gu, F. (2014). Optimized production of vanillin from green vanilla pods by enzyme-assisted extraction combined with pre-freezing and thawing. *Molecules*, *19*(2), 2181–2198.

Zhang, Z., Yu, Z., Jin, Z., Liu, J. & Li, Y. (2013). Liquid culture of adventitious roots is a potential alternative to field cultivation for *Psammosilene tunicoides*, a rare and endangered endemic medicinal plant. *Advance Journal of Food Science and Technology,* 5(2), 127-131.

Zhao, B., Agblevor, F. A., Ritesh, K. C., & Jelesko, J. G. (2013). Enhanced production of the alkaloid nicotine in hairy root cultures of *Nicotiana tabacum*. *Plant Cell Tissue Organ Culture*, *113*(1), 121-129.

Zhao, G., Fu, J., Wang, G., Ma, P., Wu, L. & Wang, J. (2010), Gibberellin-induced mesocotyl elongation in deep-sowing tolerant maize inbred line 3681-4. *Plant Breeding, 129*, 87-91.

Zhao, J., & Verpoorte, R. (2007). Manipulating indole alkaloid production by *Catharanthus roseus* cell culture in bioreactors: from biochemical prossesing to metabolic engineering. *Phytochemistry Reviews*, 6, 435-457.

Zhao, J., Davis, L. C. & Verpoorte, R. (2005). Elicitor signal transduction leading to production of plant secondary metabolites. *Biotechnology Advances, 23*, 283-333.

Zhong, J. J. (2010). Recent advances in bioreactor engineering. *Korean Journal of Chemistry Engineering*, *27*(4), 1035-1041.

Zhong, J. J., Hu, W. W., & Yao, H. U. (2001). Improvement of *Panax notoginseng* cell cultures for production of ginseng saponin and polysaccharide by high-density cultivation of pneumatically agitated bioreactors. *Biotechnol Progress*, *17*, 838-846.

Zhong, J. J., Yu, J. T., & Yoshida, T. (1995). Recent advances in plant cell cultures in bioreactors. *World Journal of Microbiology and Biotechnology, 11*, 461-467.

Zhong, Jian-Jiang. (2011). Bioreactor Engineering. Comprehensive Biotechnology book (2nd Ed.), 2, 165–177.

Zhou, L. H., Liu, S. B., Wang, P. F., Lu, T. J., Xu, F., Genin, G. M., & Pickard, B. G. (2017) The Arabidopsis trichome is an active mechanosensory switch. *Plant, Cell & Environment*, 40: 611– 621.

Zhu, C., Miao, G., Guo, J., Huo, Y., Zhang, X., Xie, J., & Feng, J. (2014). Establishment of *Tripterygium wilfordii* Hook. f. Hairy Root Culture and Optimization of Its Culture Conditions for the Production of Triptolide and Wilforine. *Journal of microbiology and biotechnology, 24*(6), 823-834.

Zhu, J., Wang, M., Wen, W., & Yu, R. (2015). Biosynthesis and regulation of terpenoid indole alkaloids in *Catharanthus roseus*. *Pharmacognosy Reviews*, *9*(17), 24–28.

Zhuo, M., Abass, O. K., & Zhang, K. (2018). New insights into the treatment of real N, N-dimethylacetamide contaminated wastewater using a membrane bioreactor and its membrane fouling implications. *RSC Advances, 8*, 12799–12807.

Zürcher, E., & Müller, B. (2016). Cytokinin synthesis, signaling, and function - advances and new insights. In Jeon K. W. (Ed.), *International Review of Cell and Molecular Biology*, *324* (pp. 1-38).Amsterdam: Elsevier Inc.