MICROPROPAGATION OF *Curcuma aeruginosa* AND *Curcuma heyneana* USING AERATED CULTURE SYSTEM

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by

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extract derived from acclimatized plantlets. (Bi) Shoot extract derived from aerated culture. (1) Bisdemethoxycurcumin. (2) Demethoxycurcumin. (3) Curcumin

LIST OF ABBREVIATIONS

BA	6-benzyladenine
DPPH	2,2- diphenyl- 1- picryl- hydrazyl
IAA	indole acetic acid
IBA	3-indole butyric acid
NAA	1-naphthaleneacetic acid
LC-MS/MS	High Performance Liquid Chromatography-Mass Spectrometry
MS	Murashige and Skoog
R_f	retention factor
t_R	retention time
TLC	Thin Layer Chromatography
UV	ultra violet
v/v	volume per volume
w/v	weight per volume
μM	micro molar

MIKROPROPAGASI *Curcuma aeruginosa* DAN *Curcuma heyneana* DENGAN SISTEM KULTUR PENGUDARAAN

ABSTRAK

Tunas rizom yang aseptik bagi *Curcuma aeruginosa* dan *C. heyneana* dapat dihasilkan dengan rendaman dalam 70% etanol selama 10 minit diikuti pensterilan permukaan dengan 20% Clorox® selama 20 minit. Teknik pensterilan ini membolehkan 73.7% dan 83.3% eksplan tunas *C. aeruginosa* dan *C. heyneana* menjadi aseptik dengan 66.7% dan 87.5% eksplan yang hidup. Masing-masing eksplan aseptik apabila dikultur dalam medium MS yang mengandungi 2.0 mg/L BA dan 0.5 mg/L NAA, sebanyak 1.9 pucuk dihasilkan daripada setiap eksplan *C. aeruginosa* manakala 2.1 pucuk dihasilkan daripada setiap eksplan *C. heyneana* selepas empat minggu pengkulturan. Eksplan pucuk *C. aeruginosa* kemudian menghasilkan lebih banyak pucuk berbilang apabila dikulturkan dalam medium cecair manakala bilangan pucuk yang dihasilkan daripada *C. heyneana* menunjukkan tidak ada perbezaan apabila dikulturkan dalam medium pepejal atau cecair. Apabila pucuk dikerat menjadi dua dan dikulturkan kedalam medium cecair, kedua-dua spesis secara bererti menghasilkan lebih banyak pucuk berbilang. Keratan pucuk daripada *C. aeruginosa* menghasilkan 4.5 pucuk per eksplan manakala *C. heyneana* menghasilkan 4.4 pucuk per eksplan selepas empat minggu pengkulturan. Eksplan pucuk daripada kedua-dua spesis dapat menghasilkan lebih kurang 250-300 pucuk di dalam balang pengudaraan 1L selepas empat minggu pengkulturan. Semua pucuk mikro kedua-dua spesis menghasilkan akar selepas dipindahkan ke medium asas MS. Kesemua pokok *in vitro* kedua-dua spesis (100%) hidup semasa diaklimitasi dengan

campuran tanah yang mengandungi tanah: pasir: tanah organik (1:1:1). Selepas 90 hari, anak pokok *C. aeruginosa* menghasilkan purata 2.3 rizom-mikro per pokok dan *C. heyneana* menghasilkan hanya satu rizom-mikro setiap pokok. Keputusan analisis TLC menunjukkan tisu yang berbeza bagi pokok *C. aeruginosa* dan *C. heyneana* menghasilkan spot yang berlainan diatas lempengan TLC yang menunjukkan penghasilan bahan yang berbeza. Analisis TLC juga menunjukkan kehadiran curcumin, demetoksicurcumin dan bisdemetoksicurcumin dalam ekstrak akar pokok *C. aeruginosa* dan *C. heyneana* yang telah diaklimitasikan. Keputusan LC-MS/MS menunjukkan anak pokok *C. aeruginosa* dan *C. heyneana* yang dihasilkan daripada sistem kultur pepejal tidak mengandungi curcumin, demetoksicurcumin dan bisdemetoksicurcumin.

MICROPROPAGATION OF *Curcuma aeruginosa* AND *Curcuma heyneana* USING AERATED CULTURE SYSTEM

ABSTRACT

Curcuma aeruginosa and *C. heyneana* aseptic rhizomatous bud explants were established with immersion in 70% (v/v) ethanol for 10 minutes followed by surface-sterilization with 20% (v/v) Clorox® for 20 minutes. This surface-sterilization technique enabled 73.7% and 83.3% of *C. aeruginosa* and *C. heyneana* bud explants became aseptic with 66.7% and 87.5% survived explants, respectively. All the aseptic explants when cultured on MS medium supplemented with 2.0 mg/l BA and 0.5 mg/l NAA produced 1.9 shoots per explant for *C. aeruginosa* and *C. heyneana* produced 2.1 shoots per explant after four weeks of culture. The shoot explants of *C. aeruginosa* subsequently produced more multiple shoots when cultured in liquid shoot proliferation medium, while the number of shoots produced from *C. heyneana* was found to be no different in the gelled or liquid medium. When the shoots were incised longitudinally into halves and cultured in the liquid medium, both species produced significantly ($p \leq 0.05$) more multiple shoot. Incised shoots of *C. aeruginosa* produced 4.5 shoots per explant while *C. heyneana* produced 4.4 shoots per explant after four weeks of culture. Shoot explants of both species could produce an approximately 250 - 300 shoots in a 1L aerated vessel after four weeks of culture. All the micro-shoots of both species formed roots after transferred onto MS basic medium. All the *in vitro* plantlets of *C. aeruginosa* and *C. heyneana* (100%) survived during the acclimatization process using soil mixture of top soil: sand:

organic soil (1:1:1). After 90 days, *C. aeruginosa* plantlets produced an average 2.3 micro-rhizomes per plantlet and *C. heyneana* produced only one micro-rhizome per plantlet. The TLC analysis results showed that different tissues of *C. aeruginosa* and *C. heyneana* produced different spots on the TLC plates which indicated production of different compounds. The TLC plate also showed that curcumin, demethoxycurcumin and bisdemethoxycurcumin were presence in the acclimatized root-tubers of *C. aeruginosa* and *C. heyneana*. The LC-MS/MS results showed that *C. aeruginosa* and *C. heyneana* plantlets derived from the *in vitro* gelled culture system did not contain curcumin, demethoxycurcumin and bisdemethoxycurcumin.

CHAPTER 1

INTRODUCTION

Medicinal plants have been used by mankind as a source of medicines since time immemorial. Approximately, 250,000 higher plant species on earth, more than 80,000 species have been reported to be used around the world for medical purposes and around 5,000 species have specific therapeutic value. Tropical countries are treasure houses of a wide variety of medicinal plants (Joy *et al*., 1998). Malaysia is ranked among the world's top 12 biodiversity rich countries where Ayurveda, Siddha, traditional Chinese, traditional Malay, Unani and other traditional systems medicine are commonly practised. More than 15,000 species of flowering plants have been reported from Malaysia and approximately 1,200 species are reported to have medicinal properties. Medicinal plants used by the Malays in the northern parts of Peninsular Malaysia have been reported (Chang and Mat Ali, 2004; Nordin *et al*., 2007). Indonesia is ranked as the 2nd biodiversity rich country in the world with 30,000 flowering plant species (Bappenas, 1993). Eisai (1986) reported that about 7,000 species have medicinal values. Plants in the Zingiberaceae family are commonly used as folk medicines in Indonesia. For example, *Zingiber officinale* (jahe) is used as anti-cancer and antiseptic agents and for the treatment of cough. *Kaempferia galanga* (kencur) also has been used for cough, *Curcuma domestica* (kunyit) as anti-diarrhoea, antiseptic and anti-cancer agents while *C. xanthorrhiza* (temulawak) are used as antihepatitis and anti-cancer agents (Bermawie, 2004).

Most of the people around the world are giving preference to the herbal medicines due to its low cost, lesser side effect and cultural beliefs (WHO, 2001).

Over three-quarters of the world population relies mainly on plants and plant extracts for health care. Traditional medicine provides an alternative option for people living in developed countries as well as in developing countries (Joy *et* al., 1998). Medicinal plants in Indonesia have high economic and health values in both indigenous and modern communities. Many research activities have reported the development of traditional medicines like 'Jamu' as standardized extracts, phytopharmaca, etc. Some of these products have been marketed and gained interest to cure major diseases such as cancer, hepatitis and heart disease (Bermawie, 2004). Popularity and demand for medicinal plants are increasing over the years within and outside Malaysia also. More intensive research with focus on natural products has been conducted in the last 20 years (Chang and Mat Ali, 2004). These data indicates the importance of medicinal plants to mankind.

Tropical monocotyledonous family Zingiberaceae consists of 50 genera and 1300 species. Mostly distributed in subtropical and warm-temperate Asia with centred in the South and South East Asia and some species are also reported from America. About 80 to 120 species of the genus Curcuma are native to tropical Asia, a few species extending to China, Australia and South Pacific. Most of the Curcuma species have been reported to be useful as medicines, spices, used for religious rituals, as dye and ornamental plants (Wu and Larsen, 2000; Škorničková, 2006).

Curcuma aeruginosa and *C. heyneana* are important medicinal plants of the genus Curcuma. The rhizomes of these species have been used in different traditional medicinal system such as Thai herbal medicine, Indonesian jamu and Malaysian traditional medicines. *C. aeruginosa* is known as blue turmeric, temu ireng or temu hitam in Indonesia and Malaysia and Waan-Ma-Haa-Mek in Thailand, while *C. heyneana* is known as pale turmeric or temu giring in Indonesia. *C.*

heyneana is distributed in Central and East Java, Indonesia (de Padua *et al*., 1999). Rhizomes of *C. heyneana* have been used as a traditional body scrub in Indonesia, locally known as "Lulur" which is famous among Indonesian women especially Javanese women for health and beauty rituals to cleanse, smoothen, soften, and rejuvenate their skin. The rhizomes also used as anthelmintic agent (Usia *et al*., 2006).

C. aeruginosa is distributed in India, Thailand, Indochina, Malaysia, Indonesia and Northern Australia (Apavatjrut *et al*., 1999). The rhizomes of *C. aeruginosa* have been used in the treatment of scabies, rheumatism, anthelmintic and obesity (Usia *et al*., 2006). The rhizomes of *C. aeruginosa* have also been reported to have antimicrobial activity, analgesic and anti-HIV properties (Phan *et al*., 2000; Murnigsih *et al*., 2005; Reanmongkol *et al*., 2006; Desai *et al*., 2009).

Rhizomes of both species were proven to have antioxidant properties as well. *C. aeruginosa* and *C. heyneana* contained curcuminoids (curcumin, demethoxycurcumin and bisdemethoxycurcumin) as antioxidant agents. These three curcuminoids have strong antioxidant activity which can replace α-tocopherol as a naturally occurring antioxidant (Jitoe *et al*., 1992). Curcumin also used as a natural colour and flavouring agent in foods, such as curry, mustard, bean cake, cassava paste and potato chips. Curcumin has also shown anti-carcinogenic activity in animals by blocking tumour initiation. Curcuminoids are important plant secondary metabolites which mainly occurred in curcuma species. However, the Curcuminoids contents vary between different species, location and cultivation period (Lin and Lin-Shiau, 2001).

Most of the species of Curcuma including *C. aeruginosa* and *C. heyneana* could be propagated vegetatively by rhizome (Škorničková, 2006). But it is time consuming to produce the rhizomes and hence unable to meet the rapid market demand. The vegetative propagation is also season dependent. Therefore, development of *in vitro* propagation technique for *C. aeruginosa* and *C. heyneana* could be the alternative for mass production of the plantlets in order to meet the market demand. Using the *in vitro* technique, large numbers of high quality, disease-free and uniform plantlets could be produced in a shorter time and without season dependency. However, micropropagation using gelled medium requires large space, gelling agents, and labour-intensive, which increase the cost of plantlets production (Berthouly and Ethienne, 2005; Takayama and Akita, 2005).

The application of bioreactor culture techniques has several advantages over conventional gelled cultures such as increase in shoot or plantlet multiplication rate, reduction in space, reduce energy and labour cost. Bioreactor vessels are generally designed for large scale tissue and organ culture in liquid medium (Paek *et al*., 2001). Considering the importance of these species, this present study is to develop an alternative propagation protocol for *C. aeruginosa* and *C. heyneana* using aerated culture system which includes the following objectives:

1) To establish aseptic cultures of *Curcuma aeruginosa* and *C. heyneana*
2) To develop a propagation protocol for *C. aeruginosa* and *C. heyneana* using aerated culture vessel
3) To determine the chemical compounds and screening for antioxidant activity of *C. aeruginosa* and *C. heyneana* plants

CHAPTER 2

LITERATURE REVIEW

2.1 The *Curcuma* species

Curcuma genus, makes up of 80 to 120 species, belongs to family Zingiberaceae. They are mostly natives to tropical Asia. A few species can be found in China, Australia and South Pacific. Curcumas are perennial rhizomatous herbs with mostly medium-sized plants about 0.5 - 1.5 m tall, the smallest species is only 10 - 20 cm and some of the species can easily reach 2 - 3.5 m. Most of the species has a pseudo stem or "false stem". The lamina or leaf blade is long and oval shape with bright to deep green above and paler green beneath. They produce large showy flowers. The bracts are mostly green to white or some with less conspicuous colour. Curcuma could be easily distinguished from other genera of Zingiberaceae family by their peculiar inflorescence (Škorničková, 2006).

Most of the curcuma species are conventionally propagated by rhizome. The rhizomes are branched, fleshy and aromatic, often with tuber-bearing roots. Externally, the rhizomes are light brown in colour but internally they have different colour shades such as yellow, blue to violet, orange, grey, cream or white (Škorničková, 2006; Wu and Larsen, 2000).

Several species of this genus are economically important due to their uses in spices, medicines, dyes, foods, perfumes, tonics and as tropical ornamentals (Chaveerach *et al*., 2008). The Curcumas are gaining worldwide importance as potential sources of new drugs that are used as anti-inflammatory,

hypocholestraemic, choleratic, antimicrobial, insect repellent, antirheumatic, antifibrotic, antivenomous, antiviral, antidiabetic, antihepatotoxic and anticancerous agents (Sasikumar, 2005).

2.1.1 *Curcuma aeruginosa*

Curcuma aeruginosa is known as temu ireng or temu hitam in Indonesia and Malaysia. It is distributed in India, Thailand, Indochina, Malaysia, Indonesia (Java and Bali) and Northern Australia (Apavatjrut *et al*., 1999; de Padua *et al*., 1999). *C. aeruginosa* is 1-2 m tall. It has green pseudo stem. The dark green to dark purplish brown leaf blade is long and oval shaped with typical burgundy midstipe. The stripes do not fade with age as much as the others curcuma species. The flowers grow from the side of the pseudo stem. The flowers are white, yellowish and some are red in colours. The rhizome is bluish-grey internally (Anon, 1978).

C. aeruginosa rhizomes are used for traditional medicine such as the treatment of rheumatism, obesity, scabies and as anthelminthic agent (Usia *et al.*, 2006). The rhizome had been also reported to have antimicrobial activity (Phan *et al*., 2000). Water extract of the rhizome inhibited the growth of *Babesia gibsoni*, a canine intra-erythrocytic parasite that causes anemia (Murnigsih *et al*., 2005). The chloroform extract possesses analgesic properties (Reanmongkol *et al*., 2006) while the aqueous extract exhibited anti-HIV properties via inhibition of HIV protease and reverse transcriptase (Desai *et al*., 2009). Only 0.02-0.03% of Curcuminoids was reported from the rhizome of *C. aeruginosa* (Bos *et al.,* 2007). The three known curcuminoids (curcumin, demethoxycurcumin, bisdemethoxycurcumin), isolated from *C. aeruginosa* rhizome, were found to have antioxidant activities (Jitoe *et al.*,

1992). Curzerenone, 1, 8-cineole [eucalyptol], furanogermenone, camphor, (Z)-3-hexenol, furanodienone, curcumenol, isocurcumenol and β-elemene are the main types of essential oils extracted from the leaves of *C. aeruginosa* (Jirovets *et al.*, 2000). However, Curzerenone, 1, 8-cineole, camphor, zedoarol, isocurcumenol, curcumenol, and furanogermanone were extracted from the rhizome (Sirat *et al.*, 1998).

2.1.2 *Curcuma heyneana*

Curcuma heyneana is a perennial rhizomatous herb with an average height of one meter. The leaf is long and narrow. The flowers are white, yellowish and some are red in colour. The rhizomes are pale yellow internally (Backer and Bakhuizen van den Brink, 1968). *C. heyneana* is known as pale turmeric or temu giring in Indonesia. It is distributed in Central and East Java, Indonesia (de Padua *et al*., 1999). The rhizome has been used as "Lulur" which is a traditional body scrub in Indonesia.

The rhizomes are used as anthelminthic against intestinal worms. The ethyl acetate-soluble fraction and methanol-soluble fraction of rhizomes showed inhibitory activity mediated by CYP3A4, which is the major hepatic and intestinal CYP in human. CYP is cytochrome P450, the main enzyme which catalyzes the metabolism of drugs and other xenobiotics (Usia *et al.*, 2006). The rhizomes also had been reported to have three known curcuminoids; namely curcumin, demethoxycurcumin and bisdemethoxycurcumin and were proven as antioxidant agent (Jitoe *et al.*, 1992). Curcuminoids content in the rhizome was found to be between 0.98-3.21% (Bos *et al.*, 2007). Zedoarondiol from the rhizome has reported to have anti-inflammatory

properties by the inhibition of iNOS, COX-2, and pro-inflammatory cytokines in LPS-stimulated murine macrophages (Cho *et al*., 2009). Isocurcumenol, 1, 8-cineole (limonene), β-eudesmol, curcumanolides A, B, dehydrocurdione and curcumenone are the composition of essential oil of *C. heyneana* rhizome (Zwaving and Bos, 1992).

2.2 Secondary metabolites

Secondary metabolites are present only in small quantity in plants and have many functions such as attractant agent and protection purpose. They help to protect the plant from microbes (bacteria, fungi, and viruses) and herbivorous (insects and vertebrates) attacks. They also help the plants to attract insects for pollination, seed dispersal, attract root nodule bacteria and induced volatiles predatory organism. Secondary metabolites also protect the plants from abiotic factors such as freezing temperatures, drought, UV light, high and low carbon dioxide, salt, metals and oxidative factors. Secondary metabolites also help the plants to adapt to the changing environment and in overcoming stress constraints (Edreva *et al.*, 2008). Until now more than 100,000 types of secondary metabolites have been isolated from plants (Hadacek, 2002). Three main groups of secondary metabolites are terpenes (29,000), alkaloids (12,000) and phenolics compounds (8,000).

Curcuma species contain curcumin, essential oil and oleoresin as the three most important secondary metabolites (Sasikumar, 2005). Curcumin is a major component of genus curcuma which has been used as yellow colouring and flavouring agent in foods. Curcumin has been proven to have anti-carcinogenic activity in animals. Curcumin is a polyphenols and they have been proven for their

role in the prevention of degenerative diseases and possess high amount of micronutrients for human diet. Polyphenols have been classified into several classes. Hydroxybenzoic acids, hidroxycinnamic acids, anthocyanins, proanthocyanidins, flavonols, flavones, flavanols, flavanones, isoflavones, stilbenes, lignans are the main classes of polyphenols (Manach *et al*., 2005). The main classes of polyphenol are naturally occurred in food stuffs and beverages (Scalbert and Williamson, 2000). Curcumin, proven to have antioxidant activity, is found in *C. aeruginosa*, *C. heyneana*, *C. domestica*, *C. mangga*, *C. xanthorrhiza* and other Zingiberaceae such as *Zingiber cassumunar*, *Phaeomeria speciosa*, *Alpinia galanga*, *Amomum kepulaga* (Jitoe *et al*., 1992).

Terpenoids is the most compound present in the essential oils of genus curcuma. Monoterpenes and sesquiterpene are terpenoids which remain constant in the essential oils. Monoterpenes include linalool, myrcene, 1.8-cineole, camphor, geraniol, curcumenol, isocurcumenol, eugenol and α pinene, while sesquiterpene are germacrone, curcumene, β-sesquiphellandrene and β-bisabolene (Sasikumar, 2005). These terpenoids are present in *C. aeruginosa*, *C. heyneana*, *C. longa*, *C. aromatica*, *C. amada*, *C. xanthorrhiza*, *C. harintha*, *C. raktakanta*, *C. pierreana*, *C. trichosantha*, *C. mangga*, *C. ochororhiza* and *C. harmandii* (Zwaving and Bos, 1992; Pham *et al*., 1994; Nguyen *et al*., 1995; Choudhury *et al*., 1996; Nguyen *et al*., 1997; Sirat *et al*., 1998; Wong *et al*., 1999; Jirovetz *et al*., 2000; Chane-Ming *et al*., 2002; Dan *et al*., 2002; Singh *et al*., 2003; Behura and Srivastava, 2004). Generally the production of secondary metabolites is affected by location and cultivation period (Lin and Lin-Shiau, 2001).

The variation profile of volatile oil in some of *Curcuma* spp. had been determined using gas chromatography (GC) or gas chromatography-mass

spectrometry (GC-MS) (Sasikumar, 2005). GC-MS and liquid chromatography-electrospray ionization mass spectrometry (LC-ESI-MS) are used to determine the chemical differences between greenhouse-grown and *in vitro* micropropagation derived plants of *C. longa* (Ma and Gang, 2006a) and *Z. officinale* (Ma and Gang, 2006b) while Ruslay *et al*. (2007) used high performance liquid chromatography coupled with diode array detection and electrospray ionization mass spectrometry (HPLC-DAD-ESI-MS) to analyze three known curcuminoids (curcumin, demethoxycurcumin and bisdemethoxycurcumin) from the rhizomes of *C. xanthorrhiza* and essential oils of *Z. zerumbet* rhizomes. The three known curcuminoids of *C. aeruginosa*, *C. domestica*, *C. heyneana*, *C. mangga*, *C. xanthorrhiza*, *Z. cassumunar*, *Phaeomeria speciosa*, *Alpinia galangal* and *Amomum kepulaga* have been analyzed using HPLC (Jitoe *et al*., 1992). Thin layer chromatography (TLC) was developed for qualitative and quantitative analysis of *C. phaeocaulis*, *C. kwangsiensis*, *C. wenyujin* and *C. longa* rhizomes (Zhang *et al*., 2008). TLC plate also had been used to isolate and separate three curcuminoids which are known to have antioxidant activity (Gupta *et al*., 1999). The antioxidant activity could be determined by thiocyanate method, thiobarbituric acid (TBA) method (Jitoe *et al*., 1992), CUPRAC method (Çıkrıkçı *et al*., 2008), screened with 1,1-diphenyl-2-picrylhydrazyl (DPPH) (Bua-in and Paisooksantivatana, 2009) and sprayed the TLC plate with DPPH (Patnibul *et al*., 2008).

2.3 Plant Tissue Culture

2.3.1 History and Developments

Based on the theory, plant tissue culture is used as a technique to propagate plants in an aseptic environment. The initial material or explants for plant tissue culture are normally selected from the healthy and actively growing mother plant. Different plant parts such as seeds, embryo, stem, shoot or root meristem or a nodal segment can be used as explants. Sodium hypochlorite, ethanol, mercury chloride or other surface sterilizing agents are used to establish aseptic explants to be cultured on a sterile medium which normally contain inorganic salts, organic vitamins and nutrients (Ahloowalia *et al*., 2004).

During 1939-1950, Street and his colleague had done extensive work on root culture to understand the role of vitamins in plant growth and shoot-root relationship. In the mid-1930s the discoveries of auxin as a natural growth regulator and the recognition of the importance of B-vitamins in plant growth led to further development of plant tissue culture techniques. The establishment of suspension cultures of plant cells in liquid medium for the production of natural plant products as an alternative to whole plant was initiated in the 1950s. The function of the auxin-cytokinin ratio was also discovered in 1950. *In vitro* culture methods were well developed in early 1960s. Hormonal manipulation in the culture medium remains the main approach to achieve plant regeneration from cultured cells and proven to be very successful with many species. Between 1970-1980, the discovery of totipotency of protoplasts and protoplasts fusion was used in crop improvement by genetic manipulation of somatic cells (Bhojwani and Razdan, 1996).

2.3.2 Micropropagation

Micropropagation is the multiplication of genetically identical plantlets by asexual reproduction. Some plants such as banana, grape, fig, double petunias and chrysanthemums produce little or no viable seeds. The only method of propagation is vegetative multiplication.

Micropropagation have several advantages over conventional methods as it requires a relatively short time, space and produces large number of plants from a single individual. To produce plantlets via micropropagation, there are various steps which include initiation of aseptic cultures, shoot multiplication, rooting and acclimatization (Bhojwani and Razdan, 1996). Micropropagation is still the most commonly used *in vitro* method for propagation of plantlets.

2.3.2.1 Establishment of Aseptic Cultures

Establishment of aseptic explants is a critical step in micropropagation. Sodium hypochlorite and ethyl alcohol are commonly used chemicals for surface sterilization of explants to establish aseptic cultures (Ahloowalia *et al*., 2004). For example, the bud explants of *Curcuma domestica* Val. and *C. xanthoriza* Roxb were sterilized by immersing in 70% ethanol for two minutes and 0.5% sodium hypochlorite for 10 minutes followed by immersing in 2% Purelox solution for 5 minutes for obtaining aseptic bud explants (Mukhri and Yamaguchi 1986). Balachandran *et al*. (1990) used 0.1% mercuric chloride solution for 15 minutes for surface-sterilization of *C. domestica* Val. var 'koova', *C. aeruginosa* Roxb. and *Zingiber officinale* Rosc. Combination of mercuric chloride and two stages of

sodium hypochlorite (surface-sterilization) were used for *C. zedoaria* and *Zingiber zerumbet* to establish aseptic bud explants (Stanly and Chan, 2007). While the buds of *Curcuma longa* Linn. were surface-sterilized with 0.07% $HgCl_2$ solution for 7-8 minutes (Shirgurkar *et al*., 2001). However Islam *et al*. (2004) surface-sterilized the *C. longa* buds by immersing them in 70% ethanol for 30-40 seconds followed by 0.1% of $HgCl_2$. While Naz *et al*. (2009) used 70% ethanol for 30-40 seconds before surface-sterilized with 20-50% sodium hypochlorite for 15 minutes. Palee and Dheeranupattana (2005) reported that sprayed *Curcuma aeruginosa* Roxb. H buds in 70% alcohol followed by 15% and 10% Clorox® solution for 15 and 10 minutes respectively, produced 66.7% aseptic survived explants.

2.3.2.2 Shoot Multiplication

In vitro shoot multiplication can be achieved through callusing, adventitious bud formation and enhanced axillary branching. Multiplication through callusing is the fastest method. However, there are several drawbacks in this method. Hence it is usually avoided in clonal propagation of a cultivar and the most serious problem is the genetic instability of callus cells. Adventitious shoot formation directly from the explants is a better approach for clonal propagation of plant species than callus method but it is restricted to only those buds that arise directly from a plant organ or tissue pieces without going through the callus phase. The enhanced axillary branching is the slowest method but with each passage the number of shoots increases logarithmically. This method is currently the most popular approach for clonal propagation of crop plants because the cells of the shoot apex are uniformly diploid and are least susceptible to genotypic changes under culture conditions.

Cytokinins are the commonly used plant growth regulator in shoot multiplication. Cytokinins in nature are involved with cell division, modification of apical dominance and shoot differentiation. However, in tissue culture it is used for differentiation of adventitious shoots from callus and organs. Benzylamino purine (BAP), isopentenyl-adenine (2-ip), furfurylamino purine (kinetin), thidiazuron (TDZ) and zeatin are cytokinins that are commonly used for this purpose. Gibberellins are rarely used but it can stimulate normal development of *in vitro* plantlets that are formed from adventives embryos (Bhojwani and Razdan, 1996).

Murashige and Skoog (1962) medium supplemented with BA (3 mg/l) could induce shoot multiplication from *Curcuma aeruginosa, C. domestica, C. caesia* and *Zingiber officinale* Roscoe shoots after four weeks of culture (Balachandran *et al*., 1990). Further study reported that MS medium supplemented with BA (1 mg/l) could induce shoot formation from *C. aeruginosa* Roxb. H bud explants after four weeks of culture (Palee and Dheeranupattana, 2005). The addition of either NAA or IBA or IAA in MS medium supplemented with BA was found to improve the shoot multiplication rate in *Z. officinale* Rosc. cv Suprava and Suruchi (Kambaska and Santilata, 2009) and *C. longa* L. cv. Ranga (Kambaska *et al*., 2010).

In vitro shoots of *C. haritha* could produce multiple shoots after 35-40 days of culture on MS medium supplemented with BA (4.4 μM) and IAA (2.9 μM) (Bejoy *et al*., 2006), while *C. longa* Linn. could produce multiple shoots on MS medium supplemented with BA (6 μM) and NAA (0.3 μM) (Islam *et al*., 2004). However Nasirujjaman *et al.* (2005) used MS medium supplemented with BA (4 mg/l) and NAA (1 mg/l) for shoot multiplication of *C. longa* L., while Panda *et al*. (2007) used MS medium supplemented with BA (3 mg/l). MS medium supplemented with BA (0.5 mg/l) and IBA (0.5 mg/l) could induce shoot

multiplication of *C. zedoaria* Roscoe and *Z. zerumbet* Smith shoots (Stanly and Chan, 2007).

Z. officinale Roscoe shoots could produce multiple shoots on MS medium supplemented with BA (2.5 mg/l) and kinetin (0.5 mg/l) (Khatun *et al*., 2003). MS medium supplemented with kinetin (3 mg/l) was the optimum medium for shoot multiplication of *Alpinia galanga* Willd (Borthakur *et al*., 1999) while MS medium supplemented with BA (1 mg/l) and IAA (0.5 mg/l) was the optimum medium for *Kaempferia galanga* L. (Parida *et al*., 2010).

2.3.2.3 Rooting

Rooting of *in vitro* shoots is required for plantlets formation. Auxins are the commonly used plant growth regulator for *in vitro* rooting. However, prolong exposure to high auxin level in the culture medium can cause callus formation, leaf chlorosis, inhibition of root elongation and dormancy in the shoot tip (Maynard *et al*., 1991). Basic MS medium was found to be effective for rooting of *Curcuma zedoaria* Roscoe and *Zingiber zerumbet* Smith (Mello *et al*., 2001; Stanly and Chan 2007). The addition of both cytokinins and auxins into the culture medium could induce the formation of normal shoots as well as roots. MS medium supplemented with 4 mg/l BAP and 1 mg/l NAA could induce multiple shoots and roots in *C. longa* Linn (Nasirujjaman *et al*., 2005), while MS medium supplemented with 3 mg/l BAP and 25 mg/l adenine sulphate could induce maximum shoot proliferation as well as root formation in *C. angustifolia* Roxb. (Shukla *et al*., 2007).

Z. officinale Roscoe (cv-Suprava and Suruchi) and *C. longa* L. (cv. Ranga) could produce roots on half-strength MS medium supplemented with NAA (2 mg/l) (Kambaska and Santilata, 2009; Kambaska *et al*., 2010). MS medium supplemented with BA (3 mg/l) could induce shoots and roots in *C. aeruginosa*, *C. domestica*, *C. caesia* and *Z. officinale* after four weeks of culture (Balachandran *et al*., 1990). Swapna *et al*. (2004) used MS medium supplemented with BA (2 mg/l) and IAA (2.5 mg/l) to induce roots for *Kaempferia galanga*, while Parida *et al*. (2010) used MS medium supplemented with BA (1 mg/l).

In tissue culture, *in vitro* and *ex vitro* rooting could be used. *Ex vitro* rooting has many advantages over the *in vitro* rooting such as reduces aseptic handling, easier and cheaper in production cost while *in vitro* rooting may be damaged during transplantation (Bhojwani and Razdan, 1996).

2.3.2.4 Acclimatization

Acclimatization is the last stage in plantlets production via micropropagation technique. Transplantation of culture from *in vitro* condition to greenhouse condition involves gradual acclimatization to achieve high plantlet survival in the greenhouse or field. Transplantation normally use a variety of potting mixes such as peat, perlite, polystyrene beads, vermiculite, fine bark, coarse sand, top soil or organic mixtures. The high survival rate of plantlets in the greenhouse is the indicator of a successful micropropagation protocol for a plant species. The plantlets need to be placed in the shaded area and maintained in moist condition for a period of time before transferring into direct sunlight condition. Application of fertilizer only begins after the plantlets were transferred into full sunlight situation (Bhojwani and Razdan,

1996). Other than the high survival rates of plantlets in field, microrhizome formation is also the indication of successful micropropagation of plants from the Zingiberaceae family.

Potting mixtures for acclimatization of *in vitro* plantlets can be different. *Curcuma zedoaria* Roscoe *in vitro* plantlets were successfully acclimatized in the pots containing a mixture of soil and rice husk ashes (3:1 ratio) (Nguyen *et al*., 2005). While acclimatization using a mixture of organic soil and sand (1:1 ratio) was found to be suitable for *C. zedoaria* Roscoe and *Zingiber zerumbet* Smith (Stanly and Chan, 2007). On the other hand, *C. longa* Linn and *Z. officinale* Roscoe can be transplanted to hydroponic culture or to pots containing soil and placed directly to the greenhouse without acclimatization (Ma and Gang, 2006a; Ma and Gang, 2006b). *C. longa* Linn also could be acclimatized in pots containing soil, cow dung and sand mixture (1:1:1 ratio) (Panda *et al*., 2007). While Naz *et al*. (2009) used mixture of sand, soil and peat (1:1:1 ratio) for acclimatizing *C. Longa* plantlets. *Kaempferia galanga* could also be acclimatized in the pots containing soil, cow dung and sand mixture (1:1:1 ratio) (Parida *et al*., 2010). A mixture of soil, sand, and manure (FYM) was used for the acclimatization of *C. longa* L. (cv. Ranga) (Kambaska *et al*., 2010) and *Z. officinale* Roscoe (cv-Suprava and Suruchi) (Kambaska and Santilata, 2009).

2.3.3 Mass propagation using aerated culture system

Micropropagation using bioreactor is a way to reduce production cost compared to the conventional techniques which are normally labour-intensive, require a large number of containers and gelified media (Berthouly and Etienne,

2005). Bioreactor is self-contained in sterile environment which capitalized in liquid nutrient for intensive culture and affording maximal opportunity to monitor and control agitation, aeration, temperature, pH, dissolved oxygen with air inflow and outflow systems. Bioreactor system can provide much more uniform culture conditions and the culture medium can be changed easily (Berthouly and Etienne, 2005). Culture system in bioreactors can be distinguished in three main types: for production of biomass (shoots or roots as the final product, cells or organogenic or embryogenic propagules), enzymes and metabolites and used for biotransformation of exogenously added metabolites (Paek *et al*., 2005).

Currently bioreactors are built in four designs for specific requirements. The four designs are the airlift and bubble column-type bioreactors, balloon-type bubble bioreactor, stirred tank bioreactor, ebb and flood bioreactor. Airlift and bubble column-type bioreactor have similar lifting system but bubble bioreactor is more suitable for large-volume cell cultivation. Circulation system provision and hydrodynamic behaviour are the major differences between airlift and bubble column bioreactors (Paek *et al*., 2001). Low shear stress, simplicity of their design and construction, availability for long-term culture with few contamination and comparatively low amount of energy consumption are the merits of the airlift type (Tanaka, 2000). Hence, airlift bioreactor is suitable for shoot mass propagation due to shoot culture more sensitive to damages caused by shear stress (Akita *et al*., 1994). Modified airlift bioreactor was adapted to aerated culture vessel.

Paek *et al*. (2001) reported that large-scale micropropagation using bioreactor had been successfully applied on several plant species such as *Lilium*, chrysanthemum, sweet potato (*Ipomoea batatas*) and Chinese foxglove (*Rehmannia glutinosa*). Bioreactor studies are leading to a possible commercial production of

secondary metabolites. Production of secondary metabolites using bioreactor also offers various advantages, e.g. controlled supply of biochemicals independent of plant bioavailability (cultivation season, pests and politics), higher yields due to well defined production systems, quality of the products are more consistent (Paek *et al*., 2005).

Airlift bioreactor reported for the production of secondary metabolites from several plant species such as *Berberis wilsonae* to produce protoberberine in 20 and 200 L (Breuling *et al*., 1985), *Frangula alnus* cell culture to produce anthraquinones (Sajc *et al*., 1995). Besides that, airlift bioreactor also reported for shoots or plantlets production of *Oxalis triangularis* (Teng and Ngai, 1999), *Ananas comosus* in 10 L (Firoozabady and Gutterson, 2003), *Spathiphyllum cannifolium* in 5 L vessel (Dewir *et al*., 2006).

Airlift has some disadvantages for mass propagation, e.g. large volumes of air induced foaming, a tendency of cells to be thrown out of the solution by bubbles or air, growth of cells in the foam at the headspace (Leathers *et al*., 1995) and they are unsuitable for high density plant cultures (Tanaka, 2000).

2.4. Chemical analysis and antioxidant activities

Antioxidants are substances that protect human body against various types of oxidative damages caused by free radicals. Hydrophilic compounds and flavonoids are the best known natural antioxidants. 2,2´-Azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) and α,α´-diphenyl-β-picrylhydrazyl radical (DPPH) are the

two most commonly used in antioxidant determination while ascorbic acid (vitamin C) and vitamin E are the two most commonly used as the positive control.

Antioxidant activity could be determined qualitatively and quantitatively in a number of different ways. The HPLC-$ABTS^{\cdot+}$ method can be used to determine hydrophilic or lipophilic antioxidant compounds, to quantify the antioxidant activity all the data expressed as trolox equivalents (TEAC) and calculated using the standard calibration curve (Arnao *et al*., 2010). Thin layer chromatography (TLC-based) qualitative antioxidant assay using DPPH spray is another method to determine antioxidant activity by the presence of a yellow, light yellow or white spot on a purple background on TLC plates, while to quantify the antioxidant activity using UV-Visible (UV-Vis) detector to record the absorbance of sample solutions which mixed with DPPH at 517 nm (Ahmed *et al*., 2007). Cousin *et al*. (2007) also used DPPH assay to quantify the antioxidant activity of *Curcuma longa* L.

Ruslay *et al*. (2007) used high performance liquid chromatography coupled with diode array detection and electrospray ion trap tandem mass spectroscopy (HPLC-DAD-ESI-MS^n) to analyze components in the antioxidant-active fractions from the rhizome of *Curcuma xanthorriza* and *Zingiber zerumbet*, while ferric thiocyanate (FTC), thiobarbituric acid (TBA) and DPPH radical-scavenging methods are used for the measurements of antioxidant activity. FTC assay is a method which measures the amount of peroxide in initial stages of lipid peroxidation while TBA assay is a method which measures the extent of peroxide degradation into malondialdehyde.

TLC had been used for the qualitative and quantitative analysis of antioxidant compounds of *Curcuma phaeocaulis*, *C. kwangsiensis*, *C. wenyujin* and *C. longa*.

The qualitative analysis using TLC plate is by viewing the TLC plate under UV 254 and 365 nm then colorized with vanillin-H_2SO_4 solution (1% vanillin dissolved in 70% H_2SO_4) and heated at 105^{o}C on a plate heater to make the spot coloured clearly and then compared the retention factor (Rf) value of samples with standard, as for the quantification the TLC plate performed densitometrically at λ_{scan} = 518 nm and $\lambda_{reference}$ = 800 nm. The content is based on the absorption spectra of samples and calculated it using the standard calibration curve (Zhang *et al*., 2008).

CHAPTER 3

MATERIAL AND METHOD

3.1 Micropropagation of *Curcuma aeruginosa* and *Curcuma heyneana*

3.1.1 Establishment of aseptic plant materials

3.1.1.1 Rhizomatous bud explants

Rhizomes from one cluster of *C. aeruginosa* and *C. heyneana* were collected from Botanical Garden, Indonesian Institute of Science (LIPI), Purwodadi, Indonesia. The bud explants of *C. heyneana* were thoroughly washed with detergent and placed under running tap water for 30 minutes. They were then immersed in 70% (v/v) ethanol for 10 minutes followed by surface-sterilization with different concentrations of Clorox® (5.3% sodium hypochlorite [NaOCl]) solution and different immersion duration (10% v/v for 10 minutes, 20% v/v for 20 minutes, 50% v/v for 10 minutes and 50% v/v for 20 minutes). The bud explants were then rinsed three times with sterile distilled water and inoculated into 350 ml glass jars containing MS basal medium (Murashige and Skoog, 1962). The pH of the medium was adjusted to 5.7-5.8 prior to autoclaving at 121° C at 1.06 kg cm^{-2} for 11 minutes. After fourteen days the percentage of aseptic buds and their survival percentage were determined. The best surface-sterilization method was then applied to *C. aeruginosa* for the establishment of aseptic bud explants. The established aseptic bud explants of both species were used for the subsequent studies.

3.1.1.2 *In vitro* seedlings

The aseptic bud explants of both species were transferred onto MS medium supplemented with 2 mg/l benzyladenine (BA) + 0.5 mg/l naphthalene acetic acid (NAA) formulated by Kambaska and Santilata (2009). The cultures were maintained at 25±2° C with continuous light provided by cool white fluorescent tube at an intensity of 35 µmol m^{-2} s^{-1}. The multiple shoots formed were used as explants for subsequent experiments. The shoots were maintained by sub culturing every 8 weeks on fresh medium with the same formulation.

3.1.2 Shoot multiplication

3.1.2.1 Shoot multiplication using MS medium supplemented with 2 mg/l BA and 0.5 mg/l NAA

Eight weeks old *in vitro* shoots of *C. aeruginosa* and *C. heyneana* were trimmed (2 cm) and inoculated into 100 ml Erlenmeyer flask containing 25 ml gelled MS medium supplemented with BA (2mg/l) and NAA (0.5 mg/l) (Kambaska and Santilata, 2009). Four *in vitro* shoots were used for each Erlenmeyer flask and 10 replicates were used for each species. The experiment was carried out using independent two sample case. All the cultures were placed in the culture room regulated at 25 ± 2^0 C and light intensity of 35 µmol m^{-2} s^{-1}. The number of shoots formed from each *in vitro* shoot was recorded after four weeks of culture. The data were analysed using student t-Test at $p \leq 0.05$.

3.1.2.2 Effect of solid and liquid medium on shoot proliferation

In order to compare the efficiency of liquid and gelled medium, eight weeks old *in vitro* shoot explants of *C. aeruginosa* and *C. heyneana* were inoculated

separately into 100 ml conical flask containing 25 ml liquid MS medium fortified with 2 mg/l BA and 0.5 mg/l NAA, shoot proliferation medium, without the addition of gelling agent. The liquid cultures were agitated on orbital shaker at 120 rpm. The shoot explants of *C. aeruginosa* and *C. heyneana* were also cultured on gelled MS medium supplemented with the same constituents. Four *in vitro* shoots were used for each Erlenmeyer flask and 10 replicates were used for each species. The experiment was carried out using 2x2 factorial design. The cultures were placed in the culture room regulated at 25 ± 2^0 C with light intensity of 35 µmol m^{-2} s^{-1}. The number of shoots form from each explant was recorded after four weeks of cultures. The data was analysed using two way ANOVA to test the combination treatment followed by Tukey's HSD Test ($p\leq0.05$).

3.1.2.3 Effect of incised and intact whole shoot on shoot proliferation

Eight weeks old *in vitro* shoots of *C. aeruginosa* and *C. heyneana* were longitudinally divided into two (half shoot) and cultured into 100 ml Erlenmeyer flask containing 25 ml liquid and gelled proliferation medium (MS medium fortified with 2 mg/l BA and 0.5 mg/l NAA). Four divided and undivided *in vitro* shoots were used for each medium in each culture vessel. Ten replicates were used for each species. The experiment was carried out using independent two sample case for each species. The number of shoots formed from each *in vitro* shoot was recorded after four weeks of cultures. The data were analysed using student t-Test at $p\leq0.05$. The cultures were placed in the culture room at 25 ± 2^0 C with light intensity of 35 µmol m^{-2} s^{-1}.