CLONAL PROPAGATION OF *Curcuma zedoaria* ROSC. AND *Zingiber zerumbet* SMITH (ZINGIBERACEAE)

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UNIVERSITI SAINS MALAYSIA

2007

CLONAL PROPAGATION OF *Curcuma zedoaria* ROSC. AND *Zingiber zerumbet* SMITH (ZINGIBERACEAE)

by

CHRISTINE

Thesis submitted in fulfillment of the requirements for the degree of Master of Science

May 2007

For my dearest family …

Acha & Amma,

&

Charles

ACKNOWLEDGEMENTS

Firstly, I thank my God for remembering me and showing an ocean of mercy on me when I was struggling through my hardest times of my life. I wish to give my heartfelt thanks and gratitude to my supervisor, Professor Dr. Chan Lai Keng whose guidance and inspiration was the key to the success of my project. I also wish to thank my co-supervisor Dr. Shahida.

I wish to thank the Universiti Sains Malaysia particularly the Dean of School of Biological Sciences and the Dean of the Institute of Higher Learning of Universiti Sains Malaysia for allowing me to pursue my higher degree. I thank the School of Biological Sciences for giving me all the facilities required for my project. My sincere thanks to the staff of the School of Biological Sciences for assisting me throughout my research especially Puan Afida, Mr. Teo, Encik Adnan Jaffer and Mr. Fasli. I thank USM library for providing unceasing resources for my project. I also wish to thank Puan Habsah for her encouragement and advice. I wish to thank all my lab mates especially Mr. Ahmed for his kindness, patience and friendship and everyone else who has been part of the team in the Plant Tissue and Cell Culture Laboratory. I also wish to thank Puan Siti for her friendship and help.

I give my sincere thanks to my dearest parents and my brother Charles for their unconditional love and support. I also wish to thank my dearest Islam for his patience and support and also for being my closest companion. Thanks to all my friends who have helped me in my research work in one way or another. I thank God again for blessing me in every possible way.

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LIST OF ABBREVIATIONS

MS	Murashige and Skoog
$HgCl_2$	Mercury chloride
BA	N_6 – Benzyladenine
IBA	Indole-3-butyric acid
IAA	Indole-3-acetic acid
GA_3	Gibberellins
NAA	1-naphthaleneacetic acid
2,4-D	2,4-dichlorophenoxyacetic acid
Picloram	4-amino-3,5,6-trichloropicolinic acid
2-ip	N_6-2-isopentyl-adenine
mm	Millimeter
cm	Centimeter
mg/L	Milligram per liter
g/L	Gram per liter
w/v	Weight per volume
v/v	Volume per volume
rpm	Rotation per minute
CRBD	Complete randomized block design
TIS	Temporary immersion system
HSD	Tukey’s Studentized Range
ANOVA	Analysis of Variance

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PROPAGASI KLON *Curcuma zedoaria* ROSC. DAN *Zingiber zerumbet* SMITH (ZINGIBERACEAE)

ABSTRAK

Untuk mendapatkan tunas rizom yang aseptik, tunas–tunas *Curcuma zedoaria* dan *Zingiber zerumbet* disterilkan dengan larutan 100 mg/L merkuri klorida ($HgCl_2$) selama lima minit, diikuti pensterilan dengan larutan 20% (v/v) Clorox® yang ditambahkan dengan beberapa titis Tween-20 selama 10 minit pada peringkat pertama dan pensterilan dengan 10% (v/v) Clorox® selama 10 minit pada peringkat kedua. Dengan protokol pensterilan tersebut, 87% tunas *C. zedoaria* yang aseptik dan mandiri boleh didapati dan 80% bagi *Z. zerumbet.* Medium proliferasi pucuk yang optimum untuk kedua-dua spesies ialah medium MS (Murashige dan Skoog, 1962) yang ditambahkan dengan 0.5 mg/L BA dan 0.5 mg/L IBA. Eksplan pucuk bagi kedua-dua spesies yang dikulturkan dalam medium proliferasi cecair dapat menghasilkan dua kali ganda bilangan pucuk berbilang berbanding dengan medium proliferasi pepejal. Tempoh masa pensubkulturan yang paling sesuai bagi spesies ini adalah lapan minggu. Pensubkulturkan secara berterusan mengkibatkan penurunan bilangan pucuk yang dihasilkan. Pucuk *C*. *zedoaria* yang dibelah dua dapat menghasilkan lebih banyak pucuk berbilang berbanding dengan pucuk sempurna. Manakala bagi *Z. zerumbet*, pucuk yang dibelah dua menghasilkan pucuk berbilang yang lebih rendah daripada eksplan pucuk yang tidak dibelah dua. Anak benih *in vitro* bagi kedua-dua spesies berjaya diaklimitisasi dengan 100% kemandirian selepas dipindah ke tanah. Eksplan pucuk *C. zedoaria* yang dibelah dua menghasilkan 3.5 pucuk per setengah eksplan pucuk setelah direndam dalam medium

proliferasi cecair selama 15 minit selama dua minggu dengan menggunakan sistem rendaman sementara (TIS). Bagi *C. zedoaria* sistem kelalang goncangan dapat menghasilkan bilangan pucuk yang lebih banyak daripada system TIS. Bagi *Z. zerumbet* pula pucuk berbilang yang dihasilkan didapati tidak berbeza secara bererti antara sistem rendaman sementara dan sistem kelalang goncangan. Bilangan pucuk *C. zedoaria* dan *Z. zerumbet* yang dihasilkan di dalam medium proliferasi yang ditambah 30 g/L sukrosa dan 15 g/L sukrosa adalah berbeza secara bererti. Walau bagaimanapun, biojism basah dan biojism kering pucuk berbilang yang diperolehi daripada kedua-dua spesies ini dalam medium proliferasi yang ditambahkan 30 g/L dan 15 g/L sukrosa didapati tidak berbeza secara bererti. Dalam sistem kelalang goncangan, bilangan pucuk, berat basah dan berat kering pucuk berbilang bagi kedua-dua spesies yang dihasilkan dalam medium proliferasi yang ditambah dengan 30 g/L dan 15 g/L sukrosa juga didapati tidak berbeza secara bererti.

CLONAL PROPAGATION OF *Curcuma zedoaria* ROSC. AND *Zingiber zerumbet* SMITH (ZINGIBERACEAE)

ABSTRACT

For establishment of aseptic rhizome buds of *Curcuma zedoaria* and *Zingiber zerumbet*, the buds were surface sterilized with 100 mg/L mercury Chloride ($HgCl_2$) for five minutes followed by 20% (v/v) Clorox® with few drops of Tween 20 for 10 minutes in the first stage and 10% (v/v) Clorox® for 10 minutes in the second stage. This sterilization protocol enabled the production of 87% aseptic and survived bud explants for *C. zedoaria* and 80% for *Z. zerumbet*. The optimum shoot proliferation medium for both the species was MS (Murashige and Skoog, 1962) medium supplemented with 0.5 mg/L BA and 0.5 mg/L IBA. Shoot explants of both species cultured in liquid proliferative medium produced twice the number of multiple shoots than solid proliferative medium. The suitable period of subculturing for these species was found to be eight weeks. Continuous subculturing of these species caused a decrease in the number of multiple shoots formed. For *C. zedoaria*, divided shoot explants produced significantly higher number of shoots than undivided or whole shoots. But for *Z*. *zerumbet* the number of multiple shoots produced by divided shoots was lower than the undivided or whole shoots. *In vitro* plantlets of both species were successfully acclimatized to the soil with 100% survival rate. The divided shoot explants of *C. zedoaria* produced 3.5 shoots per half shoots explant after being immersed in the liquid proliferative medium for 15 minutes for two weeks using temporary immersion system (TIS). For *C. zedoaria*, shake flask system produced more number of multiple shoots than TIS. For *Z. zerumbet* there was

no significant difference in the number of multiple shoots produced by TIS and shake flask system. There was significant difference in the number of multiple shoots of *C. zedoaria* and *Z. zerumbet* produced in proliferation medium (MS medium plus 0.5 mg/L BA and 0.5 mg/L IBA) plus 30 g/L and 15 g/L sucrose using TIS. However, there was no significant difference in the fresh weight and dry weight of the multiple shoots of *C. zedoaria* and *Z. zerumbet* produced in the proliferation medium plus 30 g/L and 15 g/L sucrose using TIS. In shake flask system, there was also no significant difference in the number, fresh weight and dry weight of the multiple shoots of both the species produced in proliferation medium plus 30 g/L sucrose and 15 g/L sucrose.

CHAPTER ONE

INTRODUCTION

1.1 Introduction

Plants have been utilized as medicine for thousands of years in the form of crude drugs such as tinctures, teas, poultices, powders and other herbal formulations (Balunas and Kinghorn, 2005). The medicinal properties of plants are attributed to the presence of active chemical compounds present in them. For example, Artemisinin and its derivatives are potent classes of anti-malarial drugs isolated from *Artemisia annua* which has been used in traditional Chinese medicine for centuries to treat various fevers (Picaud *et al*., 2005). Opium poppy is one of the oldest cultivated medicinal plants which were grown by Sumerians in 6000 B.C for its analgesic properties. The major alkaloids that accumulate in the latex of the poppy, codeine and morphine, are two of the most important analgesics in use today (Bradbury, 2005). Quinine from Cinchona bark was used to treat the symptoms of malaria long before the disease was identified. The raw ingredients of a common aspirin tablet have been a popular painkiller long before there was access to tablet making machinery (Gilani and Atta-ur-Rahman, 2005).

The Zingiberaceae is a moderately sized family of relatively advanced monocotyledonous plants. Species of Zingiberaceae are grown for their ornamental foliage, flowers or fruits, spices, dyes, perfumes, medicine or fiber (Neal, 1965). Members of Zingiberaceae are usually aromatic in all or most parts or at least one of the plant parts and many species are known to be rich in terpenoids. Alkaloids are also detected in Zingiberaceae species which is

an interesting finding, as alkaloids are known to be more common in dicotyledons than in monocotyledons (Larsen *et al*., 1999). Many of the Zingiberaceae plants have high medicinal value and have been used in traditional medicine for centuries. *Curcuma zedoaria* and *Zingiber zerumbet* are two important Zingiberaceae plants of high medicinal values. *Curcuma zedoaria* has been extensively cultivated as a vegetable, spice and perfume in South and Southeast Asian countries. The rhizomes of *C. zedoaria* are widely used in the treatment of ulcers, wounds, tumours, atherosclerosis and inflammation and it is prescribed in various traditional preparations used for the treatment of Ohyul syndrome, which is caused by blood stagnation (Seo *et al*., 2005). In China, the rhizomes of *C. zedoaria* are used clinically for the treatment of several types of tumours (Matthes *et al*., 1980). While *Zingiber zerumbet* has been used as a folk medicine for stomachaches, toothaches, fevers, sprains, indigestion and other ailments (Huang *et al*., 2005). The rhizomes of *Z. zerumbet* have been traditionally used as a medicine for inflammation. Zerumbone, the major component of *Z. zerumbet* was found to have chemopreventive properties (Jang *et al*., 2005).

Many medicinal plant species are disappearing at an alarming rate due to rapid agricultural and urban development, uncontrolled deforestation and indiscriminate collection. Conservation of biodiversity of medicinal plants can be achieved through *in vitro* techniques and cryopreservation (Nalawade *et al*., 2003). Tissue culture technology has often been successfully applied to the propagate plants with poor and uncertain responses to conventional methods of propagation and to preserve endangered species (Sudhersan *et al*., 2003). It also offers a potential means for conservation and mass

propagation of trees (Al-Wasel, 2000). Plant tissue culture is also important for crop and vegetable improvement using genetic engineering (Chae *et al*., 2004). Ovules-with-placental tissue culture technique is one of the effective methods of producing inter-specific hybrids between sexually incompatible lily species that are difficult to hybridize by conventional methods (Obata *et al*., 2000). Embryo rescue technique was used successfully to produce inter-specific hybrids by crossing peach as a female parent with apricot and plum (Liu *et al*., 2007). As the flowers of many herbs are resources for medical industry, it is important to reduce the time needed to reach flowering stage. Plant tissue culture can also be used as a tool for reducing juvenility and for inducing flowering, which can be done year round *in vitro* (Lin *et al*., 2003). Plant cell cultures provide an attractive route to produce high value plant derived products such as flavours, fragrances, alkaloids, colorants and pharmaceuticals that are very expensive to synthesize chemically and that occur naturally only at very low concentrations (Kim and Kim, 2002). Plant tissue culture also has an advantage of producing plants under sterile standardized conditions which are free from biotic and abiotic contaminants (Zobayed *et al*., 2004a).

Plant tissue culture techniques have been widely used for the commercial propagation of ornamental and medicinal plants. Micropropagation has proven to yield plants which are genetically identical to the donor plants. Micropropagation not only enable the production of sufficient amounts of plant material of a desired clone but it is also the basis for the future improvement through genetic engineering as well as modern germplasm conservation tasks (Tafera and Wannakrairoj, 2004). Matu *et al*. (2006) successfully established

an efficient micropropagtion protocol for the medicinally important plant *Maytenus senegalensis* and concluded that by using the protocol it was possible to generate 5000 plantlets from a single node over a period of 30 weeks. Such generated plantlets can be used to replenish the declining populations in the wild and can be cultivated by traditional medical practitioners in their gardens reducing pressure off the wild stock. Micropropagation of many members of Zingiberaceae like *Zingiber officinale (*Sharma and Singh, 1997; Khatun *et al*., 2003) *Curcuma longa* (Rahman *et al*., 2004a; Prathanturarug *et al*., 2005)*, Hydechium spicatum* (Koul *et al*., 2005)*, Kaempferia galanga* (Shirin *et al*., 2000)*, Alpinia galanga* (Borthakur *et al*., 1999), *Costus speciosus* (Malabadi *et al*., 2005) and *Aframomum corrorima* (Tefera and Wannakrairoj, 2004) had been successfully carried out before.

1.2 Research objectives

In this study, *in vitro* propagation of another two Zingiberaceae members, *Curcuma zedoaria* and *Zingiber zerumbet* were undertaken with the following objectives.

1. To establish a common shoot proliferation medium for the two species.
2. To study the effect of liquid medium on the shoot proliferation of the two species.
3. To study the effect of temporary immersion system on the shoot proliferation of both species.
4. To eventually establish an efficient micropropagation protocol for the mass production of the *in vitro* plantlets of the two species followed by successful acclimatization to the soil.

CHAPTER TWO

LITERATURE REVIEW

2.1 Zingiberaceae

2.1.1 Characteristics of Zingiberaceae family

The Zingiberaceae family consists of about 47 genera and 1400 species of perennial tropical herbs, usually in the ground flora of the lowland forests (Purseglove, 1975). The species of Zingiberaceae are widely distributed throughout India, tropical Asia, and northern Australia (Tuntiwachwuttikul *et al*., 1986). The Zingiberaceae are part of the order Zingiberales, which form an isolated group among the monocotyledons. There are about 18 genera with more than 160 species of Zingiberaceae in Peninsular Malaysia (Larsen *et al*., 1999). Zingiberaceae species grow naturally in damp and shaded parts of the lowland areas or on hill slopes, as scattered plants or as thickets. Most of the members of this family are easily recognized by the characteristic aromatic leaves and the fleshy rhizomes and also by the elliptic to elliptic-oblong leaves arranged in two rows on the leaf-shoot (Habsah *et al*., 2000). Rhizome is sympodial and fleshy and covered by scale leaves. Leaves are simple with parallel venation arranged in two rows or in a spiral, with sheath encircling the stem, with or without leafstalks and usually with a ligule.

The inflorescence may be terminal from the leafy shoot or a separate shoot from its base or from rhizome. The flowers are zygomorphic, medium sized to large and usually lasting less than twenty four hours. Calyx is tubular

with three teeth. Corolla is also tubular and slender, usually longer than calyx and three lobed, which is considered to be a modification of three stamens. One stamen of the inner whorl is fertile and the two staminoids are petal like. Anther of the fertile stamen has a dorsal connective and the pollen sacs usually dehisce longitudinally. The style is long, slender and filiform. The stigma is usually swollen. The ovary is inferior and it may be unilocular with parietal placentation or trilocular with axile placentation. The fruit is a dehiscent capsule or a fleshy berry. Seeds are usually aromatic and often arillate with the presence of perisperm and endosperm (Henderson, 1954; Purseglove, 1975; Ridley, 1967; Lawrence, 1969).

Zingiberaceae family is divided into two subfamilies. They are Zingiberoideae and Costoideae. Zingiberoideae include genera *Aframomum, Amomum, Curcuma, Elettaria, Hedychium, Kaempferia, Languas, Phaeomeria* and *Zingiber*. The subfamily Costoideae contains non aromatic plants with its centre of diversity in South America and Africa. Costoideae consist of four genera, namely *Costus, Dimerocostus, Monocostus, Tapeinochilos.* The principle genus under this subfamily is Costus (Purseglove, 1975; Larsen *et al*., 1999).

2.1.2 Importance of Zingiberaceae family

The Zingiberaceae family represent advanced monocotyledonous plants found throughout the tropical and sub tropical region with its main distribution in Asia. Generally, Zingiberaceae species are characterized by the presence of essential oils. At least 20 or more ginger species have been cultivated for their use as spices, condiments, flavours, fresh vegetables,

medicine, ornamentals and quite recently as cut flowers. The three species of Zingiberaceae of major commercial importance are *Zingiber officinale*, the fleshy branched rhizomes of which are exported to temperate countries from several tropical countries, the turmeric which is the rhizomes of *Curcuma domestica* used for colouring food and for medical purposes; and finally, *Elettaria cardomomum*, the dried capsules of which are exported as cardamom (Larsen *et al*., 1999).

Rhizomes of *Alpinia galanga, Boesenbergia pandurata* and *Kaempferia galanga* are also important members of this family which are extensively used as condiment for flavouring and as a local medicine for stomachache, carminative and diarrhoea. They are known to contain various antimicrobial agents (Oonmetta-aree *et al*., 2005, Rahman *et al*., 2004b). Rhizomes of several species of Zingiberaceae also contain insecticidal constituents. Dried and powdered rhizomes of *Curcuma longa* have been reported to be harmful to storage-pest insects such as *Tribolium castaneum*. The sesquiterpene xanthorrhizol as well as other sesquiterpenes that are present in the rhizomes of *Curcuma xanthorrhiza* are insecticidal towards larvae of the pest insect, *Spodoptera littoralis*, when applied topically via the larval integument. Rhizome extracts of *Kaempferia rotunda* and *Zingiber cassumnar* exhibited significant insecticidal activity and caused strong larval mortality or reduction in larval weight of *S. littoralis* (Nugroho *et al*., 1996).

2.1.3 Genus *Curcuma*

Curcuma, a genus of the tribe Hedychieae belonging to the family Zingiberaceae, consists of approximately 70 species. It is naturally found in

the tropical and subtropical areas, particularly in the mixed deciduous tropical forest and tropical broad leaved evergreen forest. The geographic distribution of this genus ranges from India to Thailand, Indochina, Malaysia, Indonesia and Northern Australia. The genus *Curcuma* is easily recognized by its inflorescence, a compound spike with prominent bracts each subtending a cincinnus of 2 – 10 flowers, which are joined to each other forming pouches at the base. The uppermost bracts or coma are usually the longest, generally differently coloured and can be sterile. The natural flowering seasons of *Curcuma* species varies from April to October (Apavatjrut *et al*., 1999). The genus name is derived from the Arabic word *kurkum,* referring to the yellow colour of the turmeric rhizome (Larsen *et al*., 1999; Burkill, 1966a). *Curcuma* species has a characteristic dark yellow colour and it has been found to be rich in phenolic compounds, the curcuminiods (Kim *et al*., 2006).

2.1.3.1 Botany of *Curcuma zedoaria* Rosc.

Curcuma zedoaria Rosc. which is also called zedoary, is considered to be a native of northeastern India and to have spread by cultivation throughout Indian subcontinent and Malaysia. It is called as *temu kuning* and *temu puteh* in Malaysia and Java. The rhizomes of zedoary are pale sulphur yellow to bright yellow inside, turning brownish when old. When the rhizomes are dried it has a characteristic musky odour with a slight smell of camphor and a pungent bitter taste. The main tubers are broadly ovoid with many short thick branches and tuberous roots. The leaf shoot can reach up to one meter (m) tall with about five leaves. The lamina of the leaf had a purple band on either side of the midrib when young. The inflorescence is separated from the leaf

shoot. The spike is about 16 centimeter (cm) tall with lowest bracts green, middle bracts tipped with purple and the uppermost bracts entirely purple. The flowers are pale yellow about five in the axil of each bract. Calyx is about eight millimeter (mm) long with white lobes faintly pinkish at tips. The staminode is pale yellow in colour with a concave median fold. Stamens have long and wide filament. The anthers are about six mm long with divergent and curved basal spurs (Holttum, 1950; Purseglove, 1975; Burkill, 1966a).

Plate 2.1: *Curcuma zedoaria* mother plants in their natural habitat

2.1.3.2 Chemical constituents of *Curcuma zedoaria* Rosc.

C. zedoaria contains a wide range of chemical components which have high medicinal value. The rootstock contains an essential oil, a bitter soft resin, organic acids, gum, starch, sugar, curcumin arabins, albuminoids, crude fiber and ash (Kapoor, 1990). Chemical investigations of the rhizomes reveal the presence of several sesquiterpenoids namely zederone, curcolone, curcumenol, furanodiene, curzerene, curzerenone, epicurzerenone, procurcumenol, isocurcumenol, curcumadiol, curcumol, curdione, dehydrocurdione, zingiberene, β-turmerone and ar-turmerone. It also contains the yellow colouring matter curcumin up to 0.1% (Duke *et al*., 2003; Tuntiwachwuttikul *et al*., 1986). The rhizome oil contains 1,8-cineol (18.5%), p-cymene (18.42%), α-pinene (3.28%), α-phellandrene (14.93%), terpenolene (4.11%) as major constituents (Singh *et al*., 2002). *Curcuma zedoaria* also contains other sesquiterpenoids such as curcumenone, curcumanolide A, curcumanolide B, zedoarol, 1,3-hydroxygermacrone, curzeone and zedoarondiol (Shiobara *et al*., 1986; Kouno and Kawano, 1985).

2.1.3.3 Uses of *Curcuma zedoaria* Rosc.

Zedoary is considered to have antipyretic, aphrodisiac, aromatic, carminative, demulcent, expectorant, stomachic, rubefacient and stimulant properties. It is also used as health tonic. Fresh rhizomes have diuretic properties and are used in arresting leucorrhea and gonorrheal discharges and for purifying the blood. Rhizomes are simply chewed to alleviate cough. A decoction of the rhizome administered along with long pepper, cinnamon and honey is found to be beneficial for colds, fevers, bronchitis and coughs.

Rhizomes are important ingredient of a health tonic given to women after childbirth. Externally the rhizome is applied as a paste mixed with alum, to sprains and bruises. Asian Indians apply the root to dermatitis, sprains, ulcers and wounds. Juices of the leaves are given for dropsy. Curcumol and curdione are regarded as effective anticancer compounds especially for cervical cancer and lymphosarcoma. Polysaccharide fractions of zedoary were found to decrease tumour sizes in mice and prevent chromosomal mutation. Zedoary is cultivated for starchy tubers and is used as a substitute for arrowroot and barley and is highly regarded as diet food for infants and convalescents. A red powder “Abir” which is prepared from powdered rhizomes by treatment with a decoction of sappan wood is used in Hindu religious rituals. Young rhizomes are diced and added to salads. The cores of young shoots are eaten. Fresh leaves, scented like lemongrass, are used as vegetable or for seasoning fish. It is also used in the manufacture of liqueurs, various essences, cosmetics and perfumes (Kapoor, 1990; Duke *et al*., 2003).

Morikawa *et al*. (2002) and Matsuda *et al*. (1998) reported that the aqueous acetone extract of zedoary rhizome showed hepatoprotective acitivity. The major sesquiterpenes, including furanodiene, germacrone, curdione, neocurdione, curcumenol, isocurcumenol, aerogidiol, zedoarondiol, curcumenone and curcumin were found to show protective effect against D-galactosamine / lipopolysaccharide induced acute liver injury in mice. Kim *et al*. (2005) reported that zedoary rhizome exhibited potent anti-proliferative and anti-fibrogenic effects towards human hepatic myofibroblast cells indicating that it could act as a potential medicine for treatment of chronic liver fibrosis. At 20 mg/ml, the essential oil of zedoary was moderate to good in anti-oxidant

activities, good in reducing power and excellent in scavenging effect on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical but low in chelating effect on ferrous ion (Mau *et al*., 2003). Dehydrocurdione, the major component of zedoary showed anti-inflammatory property associated to its anti-oxidant effect (Yoshioka *et al*., 1998). Dehydrocurdione also exhibited the Ca^{2+} channel blocker effect on intestinal and vascular smooth muscles of guinea pigs and rats (Irie *et al*., 2000). The chloroform and methanol extracts of zedoary rhizome showed significant analgesic activity against writhing in mice (Navarro *et al*., 2002). Water extract of zedoary rhizome showed anti-metastasic activity against the pulmonary metastasis of B16 melanoma cells (Seo *et al*., 2005). The sesquiterpenoids β-turmerone and ar-turmerone isolated from zedoary rhizome showed potent inhibitory activity of cyclooxygenase and nitric oxide synthase (Lee *et al*., 2002). Wilson *et al*. (2005) reported antimicrobial activity of zedoary against various strains of bacteria.

Zedoary was found to possess macrophage stimulating activity and has the possibility of being used as a biological response modifier (Kim *et al*., 2001). Anti-Babesial spp. activity was confirmed in an extract of the bark of zedoary rhizome. The chemical components, zedoalactones A, B, C were active against *Babesia gibsoni* (Kasahara *et al*., 2005). Zedoary showed clear inhibitory activity in the ELISA test for screening plants with antagonistic activity to *Naja naja siamensis* cobra venom (Daduang *et al*., 2005). Rhizome extract of zedoary was found to have pronounced inhibitory activity against a wide variety of human pathogenic fungi including strains resistant to the common antifungals amphotericin B and ketoconazole (Ficker *et al*., 2003). Pandji *et al*. (1993) reported that zedoary contained compounds which

showed insecticidal activity towards larvae of the vigorous pest insect, *Spodoptera littoralis*, when applied topically via the larval integument. The hexane extract of zedoary showed very strong attachment inhibiting activity against the blue mussel, *Mytilus edulis galloprovincialis* which caused marine fouling by attaching to the bottom of the ships and to the pipes in power plants causing huge economic losses (Etoh *et al*., 2003).

2.1.4 Genus *Zingiber*

Genus *Zingiber* is a moderately large genus of herbs of the family Zingiberaceae, confined to the tropics of Asia, Malaysia and the Pacific Islands (Burkill, 1966b). This genus is represented by 141 species, distributed mainly in Asia. The genus can be recognized by the presence of a pulvinus between the base of the petiole and ligule (Sabu, 2003). The inflorescence has closely overlapping bracts or bracts form an open pouch in which flowers occur, one in each bract. The whole inflorescence has a cone like appearance. In many species bracts are green when young, turning red in the fruiting stage. The flowers are usually ephemeral, only lasting a few hours. The lip of the flower is three lobed. The unique characteristic of this genus is that the stamen is attached with a long curved beak or horn like appendage. The name *Zingiber* actually comes from a Sanskrit word for bull's horn (Larsen *et al*., 1999).

2.1.4.1 Botany of *Zingiber zerumbet* Smith

Zingiber zerumbet is a perennial herb which is called as "Lempoyang" by the local people of Malaysia (Holtum, 1950). It produces light yellow flowers

in August and September. It is also called “shampoo ginger”, since the mucilaginous substance present in the inflorescence is used as shampoo and natural hair conditioner (Nalawade *et al*., 2003; Sabu, 2003). The mature flower head is saturated with a sudsy and slimy juice which was formerly used by Hawaiians for shampooing and for quenching thirst (Neal, 1965).

The perennial rhizome of *Zingiber zerumbet* is pale yellow internally. Its stem is nearly one to two meter tall. Leaves are thin about 25 – 35 cm long and sometimes purplish beneath young shoots. The midribs of the leaves are strongly raised on the lower surface. The petiole is about six mm long. The ligule is very thin, entire and broad about 1.5 – 2.5 cm long. The leaves are sheathed surrounding the stem.

The inflorescence is nearly 6 – 12 cm long, ovoid to ellipsoid in shape and green when young and becomes red when old. The bracts are about 3 – 3.5 cm long and 2.5 cm wide. The bracteole is nearly 2.5 cm long, wide and thin but persistent until fruiting. Flowers are longer than bracts, fragile and yellow in colour. The corolla tube is as long as the bract. Style is long and filiform. The stigma is slightly projecting and its margin is ciliate. One stamen of the inner whorl is fertile and the two staminoids are petal like. The stamen dehisces longitudinally. Ovary is inferior and trilocular with axile placentation. Fruit is white, thin walled, glabrous and about 1.5 cm long. The seeds are ellipsoid and black covered with lacerate aril (Holttum, 1950; Sabu, 2003).

Plate 2.2: *Zingiber zerumbet* mother plants in their natural habitat

2.1.4.2 Chemical constituents of *Zingiber zerumbet* Smith

The major chemical compound present in *Z. zerumbet* is zerumbone. Other chemical constituents include zerumbone epoxide, diferuoylmethane, feuloyl-p-coumaroylmethane, di-p-coumaroylmethane, humulene, humulene derivatives, α-pinene, camphene, caryophyllene, caryophyllene epoxide and kaempferol (Matthes *et al*., 1980). According to Chane-Ming *et al*. (2003), the essential oils from the rhizomes were rich in zerumbone, α-pinene and camphene. The essential oils obtained from the leaves and flowers contained large amounts of (E)-neridiol, β-caryophyllene and linalool. In addition to these, leaves and flowers also contain zingiberene. Jang *et al*. (2004) reported the isolation and identification of aromatic compounds p-

hydroxybenzaldehyde, vanillin and kaempferol derivatives from the chloroform ($CHCl_3$) soluble fraction of *Z. zerumbet*.

2.1.4.3 Uses of *Zingiber zerumbet* Smith

Zingiber zerumbet is used in local traditional medicine as a cure for oedema, sores and loss of appetite. The juice of the boiled rhizomes has also been used as a medicine for worm infestation in children. The aqueous extract of the plant elicited significant dose-dependent anti-inflammatory activity in mice (Somchit and Shukriyah, 2003). The rhizomes of the plant are used to relieve stomach ache and macerated in alcohol as a tonic or as a stimulant in China. In Southeast Asia, *Z. zerumbet* is traditionally used for the treatment of fever, constipation and to relieve pain. The ethanol extracts of the rhizomes of *Z. zerumbet* have shown significant analgesic and antipyretic activities in rats (Somchit *et al*., 2005). The chloroform extracts of *Z. zerumbet* have shown significant anti-giardial activity against *Giardia intestinalis*, one of the most common universal pathogenic parasites of human (Sawangjaroen *et al*., 2005). The methanol and water extracts of the plant exhibited good anti-amoebic activity against *Entamoeba histolyitca* (Sawangjaroen *et al*., 2006). While Jantan *et al*. (2005) reported that the rhizome extracts of *Z. zerumbet* showed highest platelet-activating factor receptor binding inhibitory effects on rabbit platelets. Murakami *et al*. (1999) reported that zerumbone inhibited tumour promoter 12-*O*-tetradecanoylphorbol-13-acetate-induced Epstein-Barr virus activation. Zerumbone also had a marked suppressive effect on dextran sodium sulfate-induced colitis in mice (Murakami *et al*., 2003). The dietary feeding of zerumbone isolated from *Z. zerumbet* on male F344 rats showed

significant reduction in the development of azoxy methane induced colonic aberrant crypt foci suggesting its possible chemopreventing ability (Tanaka *et al*., 2001). Humulene derivatives, 5-hydroxyzerumbone and zerumbone oxide isolated from the plant were found to inhibit lipopolysaccharide-induced nitric oxide production in murine macrophage RAW 264.7 cells (Jang *et al*., 2005). Zerumbone derivatives selectively inhibited the growth of gram positive bacteria (Kitayama *et al*., 2001). Dai *et al*. (1997) reported the HIV-inhibitory activity of zerumbone. Kim *et al*. (2004) found that zerumbone inhibited reactive oxygen and nitrogen species (RONS) generated from activated inflammatory leucocytes and inhibited mutation in AS52 Chinese hamster ovary cells. Zerumbone derivatives showed good correlation between growth inhibition of gram positive bacteria and inhibition of histidine protein kinase of YycG (Yamamoto *et al*., 2001). Zerumbone inhibited the growth of P-388D_1 cells, induced DNA fragmentation in culture and significantly prolonged the life of P-388D_1 bearing CDF_1 mice at dosage of 2mg/kg. Zerumbone also inhibited the growth of a human leukemia cell line, HL-60 cells, in a time and concentration dependent manner (Huang *et al*., 2005)

2.2 *In vitro* culture technology

2.2.1 Micropropagation techniques

Propagation of selected plant lines through tissue culture is called micropropagation (Philips *et al*., 1995). *In vitro* culture is one of the key tools of plant biotechnology that exploits the totipotency nature of plant cells, a concept proposed by Haberlandt. Tissue culture is alternatively called cell,

tissue, organ culture through *in vitro* condition. It can be employed for large scale propagation of disease free clones and gene pool conservation (Rout *et al*., 2006).

There are several steps or stages in a typical micropropagation system. It starts with Stage Zero, the selection and preparation of stock plant from which the explants are derived. In Stage One, the explant, a chosen suitable plant part, is disinfected and cultured aseptically in a culture medium. Stage Two is called multiplication phase. The objective of Stage Two is to rapidly increase the number of propagules by somatic cell embryogenesis, enhanced axillary branching or adventitious bud formation. In Stage Three, the rooting of the *in vitro* shoots is achieved. Stage Four involves hardening-off process and acclimatization of the *in vitro* plantlets to grow in the soil under greenhouse conditions for later transplanting to the field (Philips *et al*., 1995; Torres, 1989).

Stage Zero – Stock plant selection and preparation

The stock plants should be disease free, preferably maintained in either a growth chamber or greenhouse. The stock plants should be on a regularly maintained pesticide and fertilizer program. The time of the year in which the explants are taken may affect the results of micropropagation. Changes in temperature, day length, light intensity and water availability throughout a year will affect the levels of carbohydrates, proteins and growth substances in the stock plant thus subsequently affecting the response of the explant *in vitro*. Best results are generally achieved when the explant is taken during the active phase of growth. A possible exception is when the explant is derived from a storage organ. If the plant material for the explant is taken during the

dormant or resting phase of growth, the dormancy requirements may have to be met or broken. Plant material taken during the dormant or resting phase may be broken by removing the bud scales which may contain bud breaking inhibitors. Soaking shoot tips in gibberellins (GA_3) or placing the shoot in a refrigerator are techniques which may also be used to break the dormancy.

Stage One – Establishment of aseptic culture

For a successful plant tissue culture, selection of a suitable explant source is important. Almost any plant tissue can be used as an explant, but the degree of the success obtained will depend upon the culture system used, the species being cultured and the removal of surface contaminants form the explant. The main purpose of Stage One is to obtain a large percentage of explants free from surface pathogens. Disinfecting the surface generally involves washing the tissue, followed by sterilization with one or more disinfectants (Torres, 1989). There are a variety of chemical agents in common use for the surface sterilization of plant material such as calcium hypochlorite, sodium hypochlorite, hydrogen peroxide, bromine water, silver nitrate, mercury chloride and antibiotics. The choice of agent and the length of the treatment depend on the sensitivity of the material to be sterilized. Frequently it is discovered that over-zealous sterilization leads not only to the complete removal of micro-organisms, but it is also lethal to the plant tissue. Therefore it is important to determine the optimal surface sterilization conditions for each type of tissue. The sterilizing agent should be easily removable because the retention of noxious chemicals would seriously affect the growth of the tissue. Repeated washing with distilled water will remove

most chemicals. Some sterilizing agents break down and become less toxic and the products can be easily washed away. For example, sodium hypochlorite breaks down to give chlorine, the active agent, and sodium hydroxide which can be removed. The effectiveness of most sterilizing chemicals can be enhanced if a small amount of detergent like Teepol is incorporated into the sterilizing solution. The addition of detergent wets the tissue surface and allows the chemical to penetrate and destroy the micro-organisms. Another method is to rinse the tissue briefly in absolute alcohol before placing in the sterilizing agent (Street, 1973). Woody or field grown plants are sometimes very difficult to disinfect and may benefit from being placed in a beaker with cheesecloth on the top and placed under running water overnight (Beyl, 2000).

Stage Two – Multiplication of the propagules

The major goal of Stage Two is the rapid multiplication of propagules. The plant material from Stage One is repeatedly subcultured in Stage Two until the desired number of propagules or plantlets are obtained. The culture media and growth conditions are optimized for the maximum rates of multiplication (Philips *et al*., 1995). Three procedures commonly used for the clonal multiplication of plants during Stage Two are somatic cell embryogenesis, enhanced axillary development and adventitious shoot development.

Somatic cell embryogenesis

The process with the greatest potential for achieving rapid clonal micropropagation is somatic cell embryogenesis. In this process, a single cell is induced to produce an embryo, which in turn produces a complete plant. Somatic embryos are simply organized structures that originate from somatic cells but whose morphology resembles that of a developing zygotic embryo.

Enhanced axillary development

Axillary and terminal buds may be induced to develop *in vitro* by enhancing the development of quiescent or active shoot buds present. An explant containing a single bud may, depending upon the species and culture medium develop into a single shoot or produce multiple shoots. As the new shoots develop, they in turn produce buds along their axis, through repeated subculture. Callus formation may occur in association with bud development and adventitious shoots may originate from meristematic regions within the callus, thus producing more plantlets.

Adventitious shoot development

Adventitious shoots and related organs are structures that originate in tissues located in areas other than leaf axils or shoot tips. Adventitious shoots, roots, bulblets and other specialized structures may originate from stems, leaves, tubers, corms, bulbs or rhizomes. Adventitious shoots or organs may also originate from callus, which serves as an intermediate between the explant and plantlet production. The number of propagules is increased by subdividing and reculturing the *in vitro* derived organs or callus.

The plantlets or propagules attained using these techniques can be transferred to Stage Three for rooting.

Stage Three – *In vitro* rooting

In vitro-derived shoots may be induced to produce roots either *in vitro* during Stage Three or *in vivo* during stage Four. With certain species, the best results are obtained when shoots from Stage Two are transferred to a rooting medium in Stage Three. Stage Three is generally one generation in length and lasts two to four weeks during which the plantlets are rooted. But it is also known that plantlets can be rooted outside of the culture vessel and this alternative procedure is becoming more common.

Stage Four – *In vivo* rooting and acclimatization

When shoots are transferred outside the culture vessel for rooting, they must be placed in some form of medium to support their growth. Artificial soil mixtures will be referred to as rooting mixtures or soil mixtures. Rooting mixtures generally include materials such as peat, bark, perlite, vermiculite, pumice, sand and soil and may be supplemented with a small amount of lime or fertilizer. Marked differences in root formation with these various materials may be observed. The ideal rooting mixture has a neutral or slightly acidic pH and high water holding capacity, yet provides good drainage and aeration.

Acclimatization has been defined as the process by which an organism adapts to environmental change. Acclimatization is necessary because *in vitro*-derived plantlets are not adapted or suited for *in vivo* conditions. Plantlets that are to be acclimatized should be well proportioned in regards to roots and

shoots. If the plantlets are rooted *in vitro*, they generally must be transplanted to a rooting mixture and maintained under partial shade and high humidity for several days. A suitable environment generally can be created by placing the plantlets in a clear plastic bag or box or under intermittent water mist. Plants are acclimatized by gradually reducing the relative humidity in their environment. This may be accomplished by simply reducing the amount of mist the plantlets receive or by gradually slitting or opening the plastic bags or boxes that hold the plantlets.

Plants of different families or species have their own micropropagation methods which may not be suitable for other plants. Multiple shoots of *Curculigo orchiodes* were obtained from shoot tip meristem on MS medium supplemented with 2.21 µM BA and rooted on half strength MS basal medium or MS medium supplemented with 0.53 µM NAA (Wala and Jasrai, 2003). The production of complete plantlets of ash gourd, *Benincasa hispida* was achieved through multiple shoots from cotyledon derived calli (Thomas and Sreejesh, 2004). Park *et al*. (2005) developed an *in vitro* methodology for the mass propagation of *Eleutherococcus koreanum* by using adventitious root explants in liquid cultures. When the zygotic embryos of two varieties of *Zizyphus jujuba* were cultured on MS medium supplemented with varying concentration of 2,4-D, somatic embryos were formed which developed roots and shoots (Choi *et al*., 2002).

2.2.2 Media composition

Although more than 50 different media formulations have been used for the *in vitro* culture of tissues of various plants, the formulation described by

Murashige and Skoog, (1962) (MS medium) is most commonly used, often with relatively minor changes. Basically, a nutrient medium consists of all the essential major and minor plant nutrient elements, vitamins, plant growth regulators and a carbohydrate as carbon source with other organic substances as optional additives. The macronutrients nitrogen (N), phosphorous (P), potassium (K), calcium (Ca), magnesium (Mg), and sulphur (S) are required in all types of plant cultures, but the optimal concentration of each may vary with plant species. Although plant cells in culture may grow on nitrates alone, the pH of the medium (5.0 – 6.0) is usually more stable and better results are obtained when the medium contains both nitrate and ammonium ions as sources of nitrogen. The essential micronutrients which are required in trace amounts for whole plants and for tissue in culture include iron (Fe), manganese (Mn), zinc (Zn), boron (B), copper (Cu), Chlorine (Cl), molybdenum (Mo) and nickel (Ni). Iron is usually added in a chelated form to avoid precipitation and facilitate absorption. Cobalt (Co), iodine (I), and sodium (Na) are also included in some media although their requirement and physiological roles have not been established.

The carbohydrate requirement in culture media is usually met by the incorporation of 2 to 3% sucrose or less frequently by glucose. Other carbohydrates including lactose, maltose, galactose and starch have been used only rarely. In addition to their role as a carbon source, the carbohydrates act as osmotica and help maintain an osmotic potential in the culture medium that is conducive to cell and tissue growth. Whole plants synthesize all of the vitamins which act as biocatalysts, required for normal growth and development, but specific vitamins including thiamine (B1),