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IMPROVED PROPAGATION, MAXIMIZING YIELD AND ENHANCING ZERUMBONE PRODUCTION IN *Zingiber zerumbet* (L.) Smith. THROUGH SHADING AND NPK FERTILIZATION

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By

GOH SUZANNE

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

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IMPROVED PROPAGATION, MAXIMIZING YIELD AND ENHANCING ZERUMBONE PRODUCTION IN *Zingiber zerumbet* (L.) Smith THROUGH SHADING AND NPK FERTILIZATION

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Chairman : Associate Professor Thohirah Lee Abdullah, PhD
Faculty : Agriculture

Previous studies on *Zingiber zerumbet* focused mainly on chemical and biological properties of rhizome extract resulting in lack of information on cultivation practices and problems on rhizome dormancy. The determination of shade level and nitrogen (N), phosphorus (P), potassium (K) fertilizer rates are imperative for improvement on growth and rhizome yield of this high medicinal value plant. In addition to having various pharmacological traits, the species are said to be associated with a predominant compound, zerumbone (ZERU), justifying enhancement initiatives on ZERU in the species. The objectives of the present study include improvement in propagation by breaking rhizome dormancy, determination of optimum shade level and maximizing yield through NPK fertilization. The study also aimed at evaluating the inhibition effect on cytotoxic mediators of ZERU in activated microglial cells. At 100 mg/L, 6-Benzylaminopurine (BAP) and ethephon (300 mg/L) performed better in promoting breaking of dormancy in term of highest percentage of bud sprouted. Three shade levels (full sun, 30% and 50% shade levels) and five combinations of NPK fertilizer rates were tested in plant growth and yield performance. Increasing shade level and NPK fertilizer rates showed promoting pattern in plant height, number of leaves, total leaf area per plant, rhizome fresh and dry weight. Field experiment showed that 50% shade with NPK 4 (120 kg N/ha/yr, 140 kg P/ha/yr and 230 kg K/ha/yr) is the best cultural practice with highest rhizome yield (29.71 tonne per hectare) at 10 months after planting (MAP). Production of ZERU as affected by shade level and NPK fertilizer rates was evaluated using High Performance Liquid Chromatography (HPLC) with validated method. The highest concentration of ZERU (40.85% dw/dw) was from plants in pot trial grown under 50% shade and treated with NPK 3 (90 kg N/ha/yr, 105 kg P/ha/yr and 172 kg K/ha/yr). In the field experiment, ZERU decreased with increasing MAP. The highest ZERU content was found in plants harvested at 6 MAP treated with Chicken Manure and NPK 3 (90 kg/ha/yr of N, 105 kg/ha/yr of P and 172 kg/ha/yr of K) at 34.10 % (dw/dw) and 33.04 % (dw/dw) respectively. The rhizome extracts from pot trial and

ZERU were used to treat BV2-murine microglial cells with stimulation by lipopolysaccharide (LPS).The results showed selected rhizome extracts and ZERU were significantly reduced nitric oxide (NO) and reactive oxygen species (ROS) level in LPS-stimulated BV2 cells. These results showed that propagation of *Zingiber zerumbet* can be improved by application of BAP at 100 mg/L and ethephon at 300 mg/L while the yield and quality of rhizome was controlled by shading and NPK fertilization. The preliminary study on neuroinflammation showed that ZERU and extracts of rhizome has anti-inflammatory and anti-oxidant properties with significant reduction of NO level and ROS level.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

PENINGKATAN PEMBIAKAN, MEMAKSIMUMKAN HASIL DAN PENAMBAHBAIKAN PENGELUARAN ZERUMBONE DALAM *Zingiber zerumbet* (L.) Smith MELALUI RAWATAN TEDUHAN DAN PEMBAJAAN

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Kajian terdahulu ke atas *Zingiber zerumbet* tertumpu keatas sifat kimia dan biologi ekstrak rizom menyebabkan kurangnya terdapat maklumat mengenai amalan penanaman dan masalah berkaitan dengan kedormanan rizom. Penentuan ke atas tahap naungan dan pembajaan nitrogen (N), phosphorus (P), potassium (K) adalah penting bagi penambahbaikan pertumbuhan dan hasil rizom tumbuhan yang mempunyai nilai perubatan yang tinggi ini. Selain daripada mempunyai beberapa sifat farmakologi, spesies ini dikaitkan dengan satu kompaun yang dominan, zerumbone (ZERU), menjustifikasikan inisiatif penambahbaikan pengeluaran ZERU. Kajian ini telah dijalankan dengan tujuan menambahbaik pembiakan spesies dengan mengatasi keadaan dorman rizom, menentukan paras teduh yang optimum, memaksimumkan hasil melalui pembajaan NPK. Kajian juga bertujuan menilai kesan pengurangan ZERU terhadap mediator sitotoksik dalam sel-sel mikroglial aktif. Kepekatan Benzylaminopurine (BAP) pada 100 mg/L dan ethephon pada 300 mg/L member kesan yang lebih baik bagi pemecahanan kedormanan rizom dengan mempunyai peratus percambahan mata tunas yang paling tinggi. Tiga tahap naungan (penuh matahari, 30% dan 50% tahap naungan) dan lima kombinasi kadar baja NPK diuji dalam pertumbuhan dan prestasi hasil. Tahap naungan dan kadar baja NPK meningkat menunjukkan corak yang menggalakkan dalam ketinggian tumbuhan, jumlah daun, jumlah permukaan daun setiap tumbuhan, berat rizom segar dan rizom kering. Eksperimen lapangan melaporkan 50% naungan dengan NPK 4 (120 kg N/hektar/tahun, 140 kg P/ hektar/tahun and 230 kg K/ hektar/tahun) adalah amalan penanaman terbaik dengan hasil rizom tertinggi (29.71 tan setiap hektar) pada 10 bulan selepas penanaman (BSP). Penghasilan ZERU yang terjejas oleh tahap naungan dan kadar baja NPK dinilai dengan Kromatografi Cecair Prestasi Tinggi dengan kaedah yang disahkan. Kepekatan ZERU tertinggi (40.85% berat kering/berat kering) adalah daripada tanaman dalam percubaan polybags yang ditanam bawah naungan 50% dan dirawat dengan NPK 3 (90 kg N/hektar/tahun, 105 kg P/ hektar/tahun and 172 kg

K/ hektar/tahun). Dalam eksperimen lapangan, ZERU berkurangan dengan peningkatan BSP. Kandungan ZERU tertinggi ditemui di tumbuhan-tumbuhan yang dituai pada 6 BSP yang dirawat dengan tahi ayam dan NPK 3 pada 34.10% (berat kering/berat kering) dan 33.04% (berat kering/berat kering) masing-masing. Ekstrak rhizome dari percubaan polibags dan ZERU digunakan untuk merawat sel-sel mikroglial BV2 dengan rangsangan *lipopolysaccharide* (LPS). Hasilnya menunjukkan ekstrak rizom yang terpilih dan ZERU boleh mengurangkan nitrik oksida (NO) dan tahap spesies oksigen reaktif (ROS) dalam sel BV2 yang dirangsang oleh LPS. Hasil kajian menunjukkan penanaman Zingiber zerumbet boleh ditambahbaikan dengan menggunakan BAP pada 100 mg/L dan ethephon pada 300 mg/L manakala hasil dan kualiti rizom dapat dikawal dengan naungan dan pembajaan NPK. Kajian awal neuroinflammation menunjukkan bahawa ZERU dan ekstrak rizom mempunyai aktiviti anti-keradangan dan anti-oksida dengan berjaya menurunkan tahap NO dan ROS.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

µg/mL	microgram per mililiter
µM	micromolar
$µmol/m^2/s$	Micromole per meter square per second
ANOVA	Analysis of Variance
ATP	Adenosine triphosphate
BAP	6-benzylaminopurine
Ca	Calcium
Chl a	Chlorophyll a
Chl b	Chlorophyll b
CM	Chicken manure
cm/s	centimetre per second
CNB	Carbon Nutrient Balance
DCF-DA	2′,7′-Dichlorodihydrofluorescein diacetate
DEX	Dexamethasone
DMEM	Dulbecco's Modified Eagle's Medium
DMRT	Duncan Multiple Range Test
DMSO	Dimethysulphoxide
DNA	Deoxyribonucleic acid
dw	Rhizome dry weight
E	Transpiration rate
fw	Rhizome fresh weight
GCCP	Good Cell Culture Practice
G_S	Stomatal conductance
ha	hectare
HBSS	Hanks' Balanced Salt Solution

HPLC	High Performance Liquid Chromatography
K	Potassium
kg/ha/yr	kilogram per hectare per year
la	Leaf area per plant
LOD	Limit of Detection
LOQ	Limit of Quantification
LPS	Lipopolysaccharide
MAP	Months after planting
mAU	mili Arbitary Unit
Mg	Magnesium
mg/mL	microgram per mililiter
min	minute
mL	mililiter
$mol/m^2/s$	mole per meter square per second
N	Nitrogen
ng	nanogram
nl	Number of leaves per tiller
nm	nanometer
NO	Nitric Oxide
NPK	Nitrogen Phosphate Potassium
nt	Number of tiller per plant
P	Phosphorus
PA	Peak Area
PEP	Phosphoenolpyruvate
pH	Plant height
P_N	Photosynthesis rate

RNA	Ribonucleic acid
ROS	Reactive Oxygen Species
RSD	Relative Standard of Deviation
SAS	Statistical Analysis System
SE	Standard of Error
UV	Ultraviolet
ZERU	Zerumbone
ZZRE	*Zingiber zerumbet*'s rhizome extracts

CHAPTER 1

INTRODUCTION

Zingiber zerumbet (L.) Smith or known as 'Lempoyang' in Malaysia, belongs to the family Zingiberaceae. This native rhizomatous herb has been used as food flavoring in culinary preparations and the rhizomes have also been used in traditional medicine. The chemical properties and the biological activities of the rhizome extracts of Lempoyang has been widely studied. Zerumbone (ZERU) has been reported as the predominant compound in the rhizomes of *Z. zerumbet*. Various biological activities of the extracts obtained from the rhizomes were reported including: anti-inflammatory properties (Murakami, Miyamoto, & Ohigashi, 2004), anti-tumour activity (Ramos, Alía, Bravo, & Goya, 2005), preventive towards colon and skin cancer (Tanaka et al., 2001), anti-oxidant activity (Murakami et al., 2002), HIV inhibitory and cytotoxic activities (Dai, Cardellina, Mahon, & Boyd, 1997). Despite the tremendous demand for the rhizomes of *Z. zerumbet*, the agronomic and propagation practices to produce the good quality rhizomes have not been well documented.

Most of the species in the Zingiberaceae family undergo a dormancy period after each growth phase. Overcoming bud dormancy through chemical treatments can improve propagation techniques of *Z. zerumbet* and shorten the dormancy period of rhizomes. Breaking dormancy can also solve the challenges faced during dormancy such as susceptibility towards fungal diseases that further declined success rate in propagation. Application of plant growth regulator such 6-benzylaminopurine (BAP) and 2-chloroethyl phosphoric acid (ethephon) showed success in breaking dormancy in gladiolus corms (Khan, Rahman, & Hossain, 2013) and *Curcuma alismatifolia* (Thohirah, Flora, & Kamalakshi, 2010). Based on the previous literature mentioned, BAP and ethephon were believed to be able to break bud dormancy of *Z. zerumbet*.

The demand for good quality rhizome of *Z. zerumbet* is increased in Malaysia due to its potential medicinal value. However, the cultural production practices to produce good quality rhizomes are poorly documented and the commercial production of the plant is low. Lempoyang grows under full shade to partial shade areas in the lowland forest (Lim, 2014). Determination of optimum shade level is imperative to improve the growth development in this plant. Optimum light intensity can maximize the growth and yield of plant during growing period (Attridge, 1990). Rhizome yield of *Z. officinale* and *Curcuma longa* L. was reduced when growing under high shade level (Hossain et al., 2009; Kratky, Bernabe, Arakaki, White, & Miyasaka, 2013; Sreekala & Jayachandran, 2002). However, response towards shade is varied depending on the natural habitat and physiological changes of the plant. There is lack of scientific literature on the light requirement of *Z. zerumbet* for optimum growth and yield. In addition, the information on nutrient requirement for *Z. zerumbet* to produce maximum rhizome yield is lacking. Application of fertilizer is crucial and increasing fertilizer rates significantly increase the rhizome yield of *Curcuma longa* L. and *Zingiber officinale* (Akamine et al., 2007; Akhter et al., 2013). The adequate amount of fertilizer to be applied to boost rhizome yield should be known to avoid wastage and soil pollution.

The accumulation of secondary metabolites in plants is greatly affected by the environmental factors such as light intensity, nutrient availability and temperature (Akula & Ravishankar, 2011). Light intensity is the most crucial factor among all the ecological traits. Regulation of plant growth and development by light intensity was attributed by its effect on photosynthesis rate and thus this leads to synthesis of primary and secondary metabolites (Zavala & Ravetta, 2001). High light intensity were resulted in enhancement of total phenolic content in medicinal plants such as *Kaempferia parviflora* (Labrooy, Abdullah, Abdullah, & Stanslas, 2016), *Zingiber officinale* (Ghasemzadeh & Ghasemzadeh, 2011) and *Labisa pumila* (Karimi, Jaafar, Ghasemzadeh, & Ibrahim, 2013). In order to produce and obtain higher phytochemical levels in plants, suitable light intensity and nutrient requirement is necessary.

In the rhizome of *Z. zerumbet*, ZERU was found abundantly and had drawn worldwide attention to study its various effects such as anti-cancer, anti-inflammation and others (Koga, Beltrame, & Pereira, 2016). However, the potential role of ZERU and rhizome extracts to inhibit neurotoxics substances such nitric oxide (NO) and reactive oxygen species (ROS) was not evaluated so far. With the understanding of elevation in excess neurotoxics substances leads to neurodegeneration, the possible therapeutic strategy is to determine ways to suppress the release of neurotoxic substances. Hence, this study aimed to investigate the problem stated above with the general objectives:

1. To break dormancy in the rhizome of *Z. zerumbet* through pre-soaked technique using BAP and ethephon
2. To determine optimum shade levels and NPK fertilizer for better growth performance and rhizome yield at different harvesting stages
3. To enhance the production of zerumbone in rhizome of *Z. zerumbet* under optimal shade levels and NPK fertilizer rates
4. To evaluate effect of zerumbone (ZERU) and *Zingiber zerumbet*'s rhizome extracts (ZZRE) to inhibit nitric oxide (NO) and anti-oxidant properties via *in vitro* model of lipopolysaccharide (LPS)-induced brain injury using microglial BV2 cells

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LIST OF PUBLICATIONS

Thohirah, L.A., Goh, S., Hassan, S.A. and Stanslas, J. 2016. Pre-soaked technique using BAP and Ethephon in breaking bud dormancy of Lempoyang (*Zingiber zerumbet* (L.) Smith). 26th Malaysian Society of Plant Physiology Conference, MSPPC, 9-11th August 2016.

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