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MICROPROPAGATION AND DETERMINATION OF ESSENTIAL OIL COMPONENTS AND ANTIMICROBIAL ACTIVITIES OF TEMU HITAM (Curcuma aeruginosa Roxb.)

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By

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Curcuma aeruginosa Roxb. which belongs to the family Zingiberaceae is one of the oriental herbs that is gaining popularity for its medicinal properties as tonic called 'Jamu'. This species contains potential essential oils that have promising market potential with antimicrobial properties useful for food preservation, cosmetics and pharmaceutical treatment. The major constraints in the cultivation of *C. aeruginosa* are the low rate of propagation, has a period of dormancy, commercially exploited and poor flowering and seed set. Vegetative propagation through underground rhizomes often results in fungal disease that can influence the quality and quantity of essential oils within the plants. Thus, *in vitro* propagation is an alternative to accelerate plant multiplication for large scale production of true-to-type disease-free plants. The rhizome buds were tested on Murashige and Skoog medium containing BAP, Kin and TDZ at 0, 1, 2, 4, 6, 8 and 10mg/l respectively. Shoot induction was achieved after 12 weeks of culture with a maximum of 2.55 shoots on MS medium containing 1 mg/l TDZ. The excised shoots were subcultured on MS medium containing 1 mg/l TDZ with different concentrations of NAA (0, 0.1, 0.5, 1, and 2 mg/l). The shoot multiplication rate was further enhanced to 7.55 shoots on MS medium supplemented with 1 mg/l TDZ and 2 mg/l NAA after 8 weeks of culture. Rooting was tested on IBA, IAA and NAA at 0.1, 0.5, 1, 2 and 4 mg/l. Optimum rooting (26.33 roots) was obtained in MS medium containing 0.1 mg/l IAA. Well rooted shoots were acclimatized on sand, peat, soil, peat : soil (1:1), peat : sand (1:1), sand : soil (1:1) and peat : soil : sand (1:1:1). The highest plantlet survival (89%) was in mixture of peat : soil : sand (1:1:1). The essential oils and chemical composition of both field grown plants and *in vitro* grown plantlets of *C. aeruginosa* were investigated. For the field grown plant, the optimum oil yield was produced from dried rhizomes 0.63% (v/w) followed by dried leaves 0.46% (v/w), fresh rhizomes 0.26% (v/w) and fresh leaves 0.20% (v/w). The major constituents of fresh leaves oil was curzerene and germacrone (5.16% and 4.91% respectively) while dried leaves, fresh and dried rhizomes were dominated by 1,8-cineole at 17.21%, 20.53% and 18.41% and dextro-camphor at 6.55%, 6.08% and 9.89% respectively. *In vitro* grown plantlets were divided into two parts (fresh shoots and roots). The yield obtained was 0.066% (v/w) for

fresh roots and fresh shoots at 0.046% (v/w). The main group compounds in the fresh shoots oil were L-camphor (10.91%) and 1,8-cineole (6.51%) while fresh roots oil was dominated by methyl elaidate (8.99%) and methyl hexadecanoate (6.79%). The effectiveness of essential oil from different vegetative parts of *C. aeruginosa* field grown plant was tested on microorganisms. The oils from fresh leaves, dried leaves, fresh rhizomes and dried rhizomes exhibited moderate antibacterial activity against Gram positive bacteria *Staphylococcus aureus, Bacillus subtillis* and Gram negative bacteria *Eschericia coli* ranging from 7.00 to 10.00 mm. Moderate activities was also shown against two selected fungi *Aspergillus niger* and *Candida albicans* ranging from 7.00 – 9.66 mm zone of inhibition. Minimum inhibitory concentration (MIC) value to effectively inhibit the microbial activity was 0.00625 mg/µl except *S. aureus* which showed MIC at 0.0125 mg/µl. All the four extracted oils appeared to be inactive on Gram negative bacteria *Serratia marcescens*. From this study, the successful *in vitro* propagation of *C. aeruginosa* could provide large scale production of disease-free planting materials. For essential oil production, the dried rhizome of field grown *C. aeruginosa* plants were recommended as they produced more essential oil compared to leaves. Both dried and fresh rhizomes have potential as antimicrobial agent since they were dominated by 1,8-cineole and dextro-camphor which were reported to possess potent antimicrobial activity.

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

PEMBIAKAN MIKRO DAN PENENTUAN KOMPONEN MINYAK PATI DAN AKTIVITI ANTIMIKROBIAL TERHADAP TEMU HITAM (*Curcuma aeruginosa* Roxb.)

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Curcuma aeruginosa Roxb. adalah tumbuhan yang tergolong dalam keluarga Zingiberaceae. Ia merupakan salah satu tumbuhan herba ubatan yang terkenal untuk menghasilan tonik iaitu 'Jamu'. Spesies ini mengandungi minyak pati yang berpotensi untuk dipasarkan kerana ia mempunyai aktiviti antimikrob yang boleh diaplikasikan dalam pengawetan makanan, kosmetik dan rawatan farmaseutikal. Masalah utama dalam penanaman *C. aeruginosa* ialah kadar pembiakan vegetatif yang rendah, dimana ia mempunyai tempoh dorman dan kurang berbunga untuk menghasilkan benih. Selain daripada itu, pembiakan tampang menggunakan rizom sering menyebabkan penyakit kulat yang boleh mempengaruhi kualiti dan kuantiti minyak pati tumbuhan. Oleh itu, pembiakan secara *in vitro* dilihat sebagai alternatif untuk mempercepatkan pengeluaran pokok yang bebas penyakit dalam skala besar. Ujian ke atas tunas rizom telah dibuat menggunakan medium Murashige dan Skoog yang mengandungi BAP, Kin dan TDZ pada kepekatan berbeza iaitu 0, 1, 2, 4, 6, 8 dan 10 mg/l. Pengeluaran pucuk yang maksimum diperolehi dari medium MS yang mengandungi 1 mg/l TDZ selepas 12 minggu dikultur dengan jumlah pucuk iaitu 2.55. Pucuk yang telah dipotong disubkultur ke atas medium MS yang mengandungi 1 mg/l TDZ dan NAA pada kepekatan yang berbeza (0, 0.1, 0.5, 1, dan 2 mg/l). Kadar penggandaan pucuk terus meningkat kepada 7.55 dalam medium MS yang mengandungi 1 mg/l TDZ dan 2 mg/l NAA selepas 8 minggu dikultur. Pengakaran telah diuji dengan menggunakan IBA, IAA dan NAA pada kepekatan 0.1, 0.5, 1, 2, dan 4 mg/l. Medium MS yang mengandungi 0.1 mg/l IAA disyorkan untuk menghasilkan akar yang banyak (26.33 akar) dengan panjang akar iaitu 4.33 cm. Anak pokok yang telah lengkap diaklimatisasi dengan menggunakan pasir, gambut, tanah, gambut : tanah (1:1), gambut : pasir (1:1), pasir : tanah (1:1) and gambut : tanah : pasir (1:1:1). Kadar tertinggi pokok hidup (89%) diperolehi di dalam medium yang mengandungi campuran tanah gambut : tanah : pasir (1:1:1). Minyak pati dan komposisi kimia *C. aeruginosa* yang ditanam di ladang dan *in vitro* telah dikaji. Bagi pokok yang ditanam di ladang, penghasilan minyak pati yang banyak diperolehi daripada rizom kering 0.63% (v/w) diikuti oleh daun kering 0.46% (v/w), rizom segar 0.26% (v/w) dan daun segar 0.20% (v/w). Sebatian utama minyak daun segar

adalah curzerene dan germacrone (5.16% dan 4.91%) manakala daun kering, rizom segar dan kering masing-masing didominasi oleh 1,8-cineole pada 17.21%, 20.53% dan 18.41% dan dextro-camphor pada 6.55%, 6.08% dan 9.89%. Manakala, anak pokok *in vitro* dibahagikan kepada dua bahagian iaitu daun dan akar segar. Minyak pati yang diperolehi dari akar segar adalah sebanyak 0.066% (v/w) manakala pucuk segar menghasilkan sebanyak 0.046% (v/w). Sebatian utama dalam minyak pati daun segar ialah L-camphor (10.91%) dan 1,8-cineole (6.51%) manakala bagi akar segar didominasi oleh methyl elaidate (8.99%) dan methyl hexadecanoate (6.79%). Minyak pati *C. aeruginosa* yang di tanam di ladang telah diuji ke atas mikroorganisma. Minyak pati dari daun segar, kering, rizom segar dan kering menunjukkan aktiviti mikrob yang sederhana terhadap bakteria Gram positif *Staphylococcus aureus*, *Bacillus subtillis* dan bakteria Gram negatif *Eschericia coli* dengan zon perencatan antara 7.00-10.00 mm. Perencatan sederhana juga berlaku terhadap dua jenis kulat iaitu *Aspergillus niger* dan *Candida albicans* dengan zon perencatan antara 7.00 – 9.66 mm. Kepekatan minimum (MIC) yang diperlukan untuk merencatkan pertumbuhan mikrob yang diuji adalah 0.00625 mg/µl kecuali *S. aureus* yang menunjukkan nilai MIC 0.0125 mg/µl. Kesemua ekstrak daripada empat bahagian tumbuhan yang dikaji tidak menunjukkan aktiviti perencatan terhadap bakteria Gram negatif *Serratia marcescens*. Daripada kajian ini, pembiakan *C. aeruginosa secara in vitro* telah berjaya menghasilkan anak pokok yang bebas penyakit dalam skala yang besar. Bagi penghasilan minyak pati, rizom kering daripada *C. aeruginosa* yang ditanam di ladang disyorkan kerana ia menghasilkan minyak pati yang lebih berbanding bahagian daun. Kedua-dua bahagian rizom segar dan kering mempunyai potensi sebagai agen antimikrobial kerana kedua-dua bahagian ini didominasi oleh 1,8-cineole dan dextro-champor yang dilaporkan mempunyai aktiviti antimikrob yang berkesan.

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This is to comfirm that:

- the research conducted and the writing of this thesis was under our supervision;
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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
BAP	6-Benzylaminopurine
cm	Centimetre
DMRT	Duncans Multiple Range Test
et al.	Et alia
g	Gram
g/l	Gram per litre
H	Hour (s)
IBA	Indole-3-butyric acid
IAA	Indole-3-acetic acid
KIN	Kinetin
l	Litre
mg	Milligram per liter
mm	Millimetre
µmol m^{-2} s^{-1}	Micromole per meter square per second
µM	Micromolar, 10^{-3} mM
MS	Murashige and Skoog
NAA	α-Naphthalene acetic acid
PGR	Plant growth regulator
pH	-log (H+)
RCBD	Randomized Complete Block Design

sp.	Species
TDZ	Thidiazuron
µl	Microlitre
%	Percent
^{o}C	Degree centigrade
v/w	Volume per weight

CHAPTER 1

INTRODUCTION

1.1 Background

Turmeric or "kunyit" (*Curcuma longa*) is one of the most important economic spices in Malaysia. *Curcuma aeruginosa* has been traditionally used as ingredient in tonic called 'Jamu' (Mohd Aspollah *et al*., 2007), treatment for bruises and sprains (Mandal *et al*., 2013), treatment for skin cuts, scrapes, acnes, diaper rash and psoriasis, treatment to reduce inflammation and redness (Pyo and Oo, 2007) and alleviation to painful female menstruation (Thaina *et al*., 2009). This plant species also scientifically proven to have an androgenetic treatment for increased hair growth and slowed hair loss in men (Pumthong *et al*., 2012), anti skin-ageing properties in post-menopausal woman (Yingngam *et al*., 2011), antimicrobial activities, antioxidant properties and anti-inflammatory properties (Mandal *et al*., 2013).

In Malaysia, people have changed their preferences from using synthetic products to natural products for medicinal and cosmetic purposes since they are safe and more reliable. The medicinal property of the plant is exhibited from the chemical compounds found in the plant extract such as in the essential oil. The essential oil contained thousands of different compounds which are important for therapeutic industry. The essential oil of *C. aeruginosa* has been used as ingredient in traditional folk medicine. However, the therapeutic properties were poorly understood especially the pharmacology effect it possessed. Thus, scientific investigation is needed to provide scientific validation for its traditional uses.

Despite its beneficial values, vegetative production of *C. aeruginosa* is not feasible due to its low propagation rate (Mandal *et al*., 2013), existence of dormancy phase (Palee and Dheeranupattana, 2005) and highly susceptibility to diseases (Kambaska *et al*., 2010). Moreover, excessive collection from the wild due to market demand caused plant materials source decrease.

In order to overcome these problems, tissue culture is seen as an alternative method to ensure sustainable large scale production of planting materials in a short period of time. Moreover, further investigations to determine the chemical composition of the essential oil and antimicrobial activities for *C. aeruginosa* are needed.

1.2 Research Objectives

To produce planting materials in large quantity using *in vitro* techniques and to determine the chemical constituents and antimicrobial activity of the essential oils extracted from different parts of in vivo and *in vitro* plants of *C. aeruginosa.*

The specific objectives were:

1. To determine the effects of plant growth regulators on shoot induction, shoot multiplication and rooting in *C. aeruginosa*.
2. To determine a suitable potting medium for acclimatization.
3. To determine the essential oil constituents of field grown plants and *in vitro* grown plantlets of *C. aeruginosa*.
4. To determine the antimicrobial activity of the essential oil of *C. aeruginosa.*

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