

**ANTIFUNGAL AND AUXIN-LIKE ACTIVITY OF CARBENDAZIM
IN MICROPROPAGATION AND ESSENTIAL OIL COMPOSITION
OF *EUCALYPTUS CAMALDULENSIS* DEHNH.**

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**THESIS SUBMITTED IN FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY**

**FACULTY OF SCIENCE
UNIVERSITY OF MALAYA
KUALA LUMPUR**

2015

UNIVERSITI MALAYA

ORIGINAL LITERARY WORK DECLARATION

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MICROPROPAGATION AND ESSENTIAL OIL COMPOSITION OF
EUCALYPTUS CAMALDULENSIS DEHNH.**

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ABSTRACT

An investigation on *Eucalyptus camaldulensis* Dehnh. was carried out using samples from matured field grown trees in Malaysia. Surface sterilization and callus induction protocols have been described intended for *in vitro* plant regeneration using leaf and stem explants. A multi-option surface sterilization protocol has been established using three common sterilizing agents such as Mercuric Chloride (HgCl₂), Sodium Hypochlorite (NaOCl) and ethanol which were tested by varying their combinations and their time of exposure. Combined treatments with two and three of sterilizing agents proved efficient in getting higher rates of clean, especially, when 70% alcohol was included. Using of 2.63% NaOCl for 10 min, 0.1% HgCl₂ for 2.0 min and 70% ethanol for 1.0 min was the most effective sterilizing method for *E. camaldulensis* leaf explants and could obtain low contamination and damage rates, which can be applied to other plants with the same rigidity.

Callus induction was successfully obtained on MS medium from leaf and stem explants using different auxins and cytokinins i.e. 6-Benzylaminopurine (BA), Kinetin (Kn), 2,4-Dichlorophenoxyacetic acid (2,4-D), Indole-3-acetic acid (IAA), Indole-3-butyric acid (IBA) and 1-naphthaleneacetic acid (NAA) in the concentration range of 0.5 - 3.0 mg/l. IBA and 2, 4-D were shown to be the best hormones for callus induction in leaves and stems, respectively. Combination of BA and NAA showed a better callus induction response, than individual hormones. Embryogenic callus was produced in leaf explants using IBA and the globular and heart stages were observed.

The effects of the potent fungicide Carbendazim (MBC) were evaluated in *in vitro* cultures of *E. camaldulensis*. It showed significant reduction in fungal contamination, especially at ≥ 25 mg/l and low phytotoxic effects. Callus induced by NAA and BA from leaf and stem explants were not highly sensitive to MBC at low and moderate concentrations (≤ 15 and < 50 mg/l, respectively). At higher MBC concentrations, callus

induction and organogenesis were decreased. The highest MBC concentrations (200 and 300 mg/l), inhibited callus induction in leaf and callus development in stem. In addition to its cytokinin-like effect, the compound showed auxin-like effect by enhancement of callus induction in stem explants. The remarkable callus induction increment was observed when 3 mg/l MBC was combined with 3 mg/l 2,4-D or 0.1 mg/l BA and 3.0 mg/l NAA. Stem callus derived from MBC grown cultures could be efficient, regenerated into complete plants and established in field conditions. Direct shoot formation using plant nodal segments was also not sensitive to the fungicide concentrations (≥ 50 mg/l). Increasing concentrations of MBC in media caused some ultrastructural and anatomical alterations. Thus, these findings may have significance towards the understanding of the roles of the fungicide in plants.

The essential oils compositions of *E. camaldulensis* various organs were investigated. Fifteen (15) major compounds were identified represented 98.8, 97.5 and 99.5% of the compounds in the leaves, stems and immature flowers, respectively. Monoterpenes hydrocarbons were predominant in the three oil samples. The flower oil had the highest oxygenated monoterpenes content (19.6%). The most abundant compound in the three essential oils was γ -terpinene, as a principal oil component (57.4–72.5%) followed by *o*-cymene (14.6–26.3%) and terpinen-4-ol (6.6–16.2%). The regenerated plants from callus induced in stem using 3.0 mg/l NAA and 0.1 mg/l BA and 3.0 mg/l MBC, rooted on MS half strength medium fortified with 1.0 mg/l IBA and successively established under the field environment. They showed the same essential oil major components as those of the field-grown plants. In addition, *E. camaldulensis* leaf oils demonstrated cytotoxic effects in four cell lines; WEHI-3, HT-29, HL-60 and RAW 264.7 with WEHI-3 was the most sensitive to the essential oils with IC₅₀ 16.1.

ABSTRAK

Satu kajian mengenai kultur tisu, antikulat dan komposisi minyak pati ke atas *Eucalyptus camaldulensis* Dehnh. telah dijalankan dengan menggunakan sampel daripada pokok matang yang ditanam di Malaysia. Efektif protokol ke atas pensterilan eksplan dan induksi kalus telah dijalankan untuk regenerasi tumbuhan *in vitro* menggunakan eksplan daun dan batang. Beberapa kombinasi protokol pensterilan telah berjaya dikenalpasti dengan menggunakan tiga agen pensterilan biasa iaitu merkuri klorida, natrium hipoklorida dan etanol dengan mengubah gabungan kepekatan dan tempoh persentuhan eksplan dengan larutan pencuci. Menggabungkan dua dan tiga agen pencucian dalam pencucian eksplan adalah lebih berkesan dalam mendapatkan kadar kultur tanpa jangkitan mikroorganisma yang lebih tinggi terutamanya apabila 70% etanol digunakan. Pensterilan dengan menggunakan 2.63% NaOCl selama 10 min, 0.1% HgCl₂ selama 2 min dan 70% etanol selama 1 min adalah paling berkesan untuk mensteril eksplan daun *E. camaldulensis* di mana kadar kontaminasi dan kerosakan yang diperhatikan adalah rendah. Protokol pensterilan ini memungkinkan ia diaplikasikan ke atas tumbuhan lain yang mempunyai ketegaran yang sama.

Induksi kalus berjaya diperolehi dalam MS media untuk eksplan daun dan batang dengan menggunakan pelbagai jenis auksin dan sitokinin iaitu 6-Benzylaminopurine (BA), Kinetin (Kn), 2,4-Dichlorophenoxyacetic acid (2,4-D), Indole-3-acetic acid (IAA), Indole-3-butyric acid (IBA) dan 1-naphthaleneacetic acid (NAA) dalam julat kepekatan 0.5 - 3.0 mg/l. IBA adalah hormon yang terbaik untuk induksi kalus pada eksplan daun manakala 2,4- D adalah hormon yang terbaik untuk induksi kalus pada batang. Kombinasi BA dan NAA menunjukkan tindak balas yang lebih baik bagi induksi kalus berbanding hormon digunakan secara individu. IBA telah menghasilkan kalus embriogenik dan beberapa peringkat embriogenesis somatik telah diperhatikan.

Kesan racun kulat carbendazim telah diperhatikan di dalam kultur *in vitro* *E. camaldulensis*. Sebatian ini menunjukkan pengurangan pencemaran kulat yang signifikan terutamanya pada 25 mg/l dan kesan fitotoksikiti yang rendah. Kalus daripada daun dan batang *E. camaldulensis* dihasilkan oleh NAA dan BA tidak begitu sensitif kepada MBC pada kepekatan yang rendah dan sederhana (masing-masing bernilai ≤ 15 dan ≤ 50 mg/l). Pada kepekatan MBC yang lebih tinggi, induksi kalus dan organogenesis berkurang. Sementara itu, pada kepekatan tertinggi MBC (200 dan 300 mg/l), induksi kalus direncat daripada eksplan daun dan batang. Selain kesan seperti sitokinin, carbendazim juga menunjukkan kesan seperti auksin melalui peningkatan induksi kalus daripada eksplan batang. Peningkatan induksi kalus yang ketara telah diperhatikan apabila 3.0 mg/l MBC digabungkan dengan 3.0 mg/l 2,4-D atau 0.1 mg/l BA dan 3.0 mg/l NAA. Kalus batang yang di berikan MBC boleh dipropagasi untuk menghasilkan tumbuhan lengkap sepertimana tumbuhan yang tumbuh di kawasan lapangan. Organogenesis langsung menggunakan batang bernod juga tidak sensitif pada kepekatan MBC bernilai 50 mg/l. Oleh itu, penemuan ini mungkin mempunyai kepentingan yang signifikan ke arah memahami peranan racun kulat ke atas tanaman.

Komposisi minyak pati daripada pelbagai bahagian tumbuhan *E. camaldulensis* telah dikaji. Lima belas (15) sebatian utama telah dikenalpasti di mana masing-masing mewakili 98.8, 97.5 dan 99.5% daripada sebatian dalam daun, batang dan bunga yang tidak matang. Monoterpena hidrokarbon adalah komponen utama dalam tiga sampel minyak pati. Minyak pati bunga mengandungi sebatian 'Oxygenated monoterpene' yang tertinggi (19.6%). Sebatian yang paling banyak terdapat dalam tiga minyak pati adalah γ -terpinene, sebagai satu komponen asas minyak pati (57.4-72.5%) diikuti oleh *o*-cymene (14.6-26.3%) dan terpinen-4-ol (6.6-16.2%). Tumbuh-tumbuhan yang dihasilkan daripada kalus batang yang diinduksikan oleh 0.1 mg/l BA dan 3.0 mg/l NAA dan 3.0 mg/l MBC menghasilkan akar pada media MS berkepekatan separuh yang ditambah

dengan 1.0 mg/l IBA. Tumbuhan yang telah berjaya di pindahkan ke tanah kemudian dikaji komposisi minyak patinya. Tumbuh-tumbuhan hasil kultur menunjukkan komponen utama yang sama seperti yang terdapat pada pokok yang terdapat di lapangan. Minyak pati daripada daun *E. camaldulensis* menunjukkan kesan sitotoksik dalam empat strain sel; iaitu sel WEHI-3, HT-29, HL-60 dan RAW 264.7. WEHI-3 adalah yang paling sensitif terhadap minyak pati dengan nilai IC_{50} 16.1.

ACKNOWLEDGEMENTS

All praises and thanks to the Almighty God, ALLAH, The most merciful and the most compassionate, who created man and taught him manners and is the source of entire Knowledge and Wisdom endowed to mankind.

I pay the sense of gratitude to my supervisor Prof. Dr. Rosna Mat Taha, Coordinator for Biotechnology Programme, Institute of Biological Sciences, University of Malaya, Kuala Lumpur for her dynamic supervision, propitious guidance, valuable advices, keen interests and useful suggestion throughout the research endeavor.

The author is obliged to University of Malaya, Kuala Lumpur and International University of Africa, Sudan for facilities and financial supports.

Heartiest thanks to my brother and friend Dr. Bakrudeen Ahmed, Programme of Biotechnology for his critical insights, valuable advices and helps.

I am also thankful to all my colleagues and research fellows in the Biotechnology laboratory, especially, Dr. Sadegh Mohajer and all my well-wishers who have helped me by their moral supports throughout my educational tenure.

Finally, I would like to express my humble obligations to my family members for their prayers, wholehearted encouragements, and providing conditions favorable to accomplish my academic goals.

(Elnaiem Elaagib Mubarak)

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LIST OF ABBREVIATIONS

2, 4-D	:	2,4-dichlorophenoxyacetic acid
ANOVA	:	Analysis of Variance.
B5	:	Gamborg Medium
BA	:	6-Benzylaminopurine, Benzyl adenine.
C ₈ –C ₂₀	:	n-alkane series of C ₈ –C ₂₀ .
CRD	:	Complete Randomized Design.
DMEM	:	Dulbecco's modified Eagle's medium
DMRT	:	Duncan's multiple range test
DMSO	:	Dimethyl Sulfoxide
ELISA	:	Enzyme-linked immunosorbent assay.
FAA	:	Formaldehyde – acetic acid – ethanol solution
FBS	:	Fetal bovine serum
g/l	:	Gram per liter.
GA ₃	:	Gibberellic acid
GC-FID	:	Gas chromatography/flame ionization detector
GC-MS	:	Gas chromatography/mass spectrometry
IAA	:	Indole-3-acetic acid
IBA	:	Indole-3-butyric acid
IC ₅₀	:	Half maximal inhibitory concentration.
Kn	:	Kinetin; 6-furfurylaminopurine
LC ₅₀	:	The lethal concentration, 50%.
MBC	:	Methyl 1-2-benzimidazole carbamate, carbendazim
mg/l	:	Milligram per liter
MS	:	Murashige and Skoog medium, (Murashige and Skoog, 1962)

MTT	:	3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide
NAA	:	1-Naphthaleneacetic acid.
NIST	:	National Institute of Standards and Technology.
OD	:	Optical density (absorbance).
p-CPA	:	p-chlorophenoxyacetic acid
PGR	:	Plant Growth Regulators
RI	:	Retention index
RT	:	Retention time.
SE	:	Standard Error.
SEM	:	Scanning Electron Microscope.
WPM	:	Woody Plant Medium.