UNIVERSITI PUTRA MALAYSIA

ISOLATION, SCREENING FOR BIOACTIVITIES AND IDENTIFICATION OF SELECTED ENDOPHYTE FUNGI BY SEQUENCING OF 18S rRNA/ITS GENES

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By

CHEAH YOKE KQUEEN

Thesis Submitted in Fulfilment of the Requirement for the Degree of Master of Science in the Faculty of Food Science and Biotechnology Universiti Putra Malaysia

August 2001

DEDICATION

To my mother, Sheon Yoke Chun and

my father, Cheah Seak Yuen for their help

and prayers……

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

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Chairman : Associate Professor Son Radu, Ph. D.

Faculty : Food Science and Biotechnology

Endophytic fungi occur within plant tissues such as leaves and stems of healthy plants without producing any apparent infections and symptoms. Experiments were conducted to isolate endophytic fungi from healthy medicinal plants as well as detecting any reoccurrence of any particular endophytic fungi predominantly in selected local medicinal plants. Endophytic fungi have also been recognised as repository of novel secondary metabolites, which have beneficial biological activities, biocontrol of insects and oligosaccharides degrading enzymes. Thus, isolated endophytic fungi were screened for their bioactive properties by using Thin Layer Chromatography and Agar Diffusion Assay. Specially engineered yeast strains i.e. UCS and UCK from Kyowa Hakko Company, Japan were used to screened for anti-tumour activity. 18S ribosomal Ribonucleic Acid (18S

rRNA) / Internal Transcribed Spacer (ITS) gene sequencing were conducted to identified certain isolates.

Isolation of the endophytic fungi from healthy local medicinal plants showed that 61 out of 72 (84.7%) yielded endophytic fungi. Most of the endophytes were obtained from the leaves and very few from the stems. The reoccurrence rate of the endophytic fungi was 1.39% (1 in 72). Nevertheless, no predominant endophytic fungi association with types of medicinal plant was observed.

All the isolated endophytic fungi were able to degrade starch, xylan, mannan and inulin. 98.33% of the endophytic tested were able to degrade sago starch and rice starch. About 96.67% of the isolates were detected producing potato starch and starch wheat unmodified degrading enzyme. However, 95% of the endophytes produced tapioca starch and corn starch degrading enzyme.

Among the isolated endophytic fungi, 16.9% of them were considered important with regard to the bioactive screening results. Thus, 22 isolates from 130 isolated fungi gave satisfactory results in bioactive screening. There were 16 isolates that gave positive results in bio-activity test against UCS/UCK yeast strain. This showed that 16 out of 130 isolates produced potential bioactive compound for anti-tumour.

Four out of the twenty two important isolates were identified through microscopic examination and 18S/ITS gene sequencing. Isolate 12L was

identified as *Penicillium* spp. through microscopic morphology observation. Those isolates identified by 18S/ITS gene sequencing included 1B, *Endothia gyrosa*; 19L, *Colletotrichum gloeosporioides* and 22L, *Botryosphaeria ribis* . BLAST programme was used as a tool to determine the homology of the sequence obtained with the database sequence.

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

PEMENCILAN, PENSKRINAN BIOAKTIVITI DAN IDENTIFIKASI "ENDOPHYTE FUNGI" YANG TERPILIH DENGAN PENJUJUKKAN GEN '18S/ITS'NYA

Oleh

CHEAH YOKE KQUEEN

Ogos 2001

Pengerusi : Profesor Madya Son Radu, Ph. D.

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"Endophytic fungi" terdapat di antara tisu tumbuhan umpamanya tisu, daun dan batang tumbuhan yang sihat tanpa menyebabkan sebarang infeksi dan simptom-simptom yang ketara. Eksperimen dijalankan untuk mengisolatkan "endopytic fungi" daripada tumbuhan berubat yang sihat dan mengenalpasti sebarang kehadiran semula "endophytic fungi" secara pradominan dalam tumbuhan berubat tempatan yang terpilih. "Endophytic fungi" juga di kenali sebagai tempat penyimpan metabolit sekunder yang bermanfaat dari segi aktiviti biologi, kawalan biologi terhadap serangga dan enzim pencernaan oligosakarid. Dengan demikian, "endophytic fungi" yang dipencilkan itu diskrinkan terhadap unsur bioaktif dengan "Thin Layer Chromatography" dan "Agar Diffusion Assay". Yeast yang khas dijurutera i.e. UCS dan UCK dari syarikat Kyowa Hakko digunakan untuk penskrinan unsur bioaktif antitumor.

Penjujukkan gen "18S ribosomal ribonucleic acid" (18S rRNA) / "Internal Transcribed Spacer" (ITS) dijalankan untuk mengenalpasti pencilan-pencilan tertentu.

Pemencilan "endophytic fungi" daripada tumbuhan berubat yang sihat menunjukkkan 61 daripada 72 (84.7%) terdapatnya "endophytic fungi". Kebanyakkan "endophytes" yang diperolehi adalah daripada daun dan sangat jarang daripada batang ataupun tangkai. Kadar kehadiran semula "endophytic fungi" adalah 1.39% (1 in 72). Walaubagaimanapun, tiada "endophytic fungi" yang pradominan berkait dengan sebarang jenis tumbuhan yang dikaji kelihatan.

Semua pencilan "endophytic fungi" berupaya menurunkan kanji, xylan, mannan dan inulin. 98.33% daripada "endophytic fungi" yang dikaji berupaya menurunkan kanji sago dan kanji beras. Lebih kurang 96.67% daripada pencilan dikesan menghasilkan enzim penurunan kanji kentang dan "starch wheat unmodified". Namun demikian, 95% daripada "endophytes" menghasilkan enzim penurunan kanji ubi dan kanji jagung.

Di kalangan pencilan-pencilan "endophytic fungi", 16.9% daripadanya di katakan mustahak berhubungan dengan penskrinan bioaktif. Oleh itu, 22 pencilan daripada 130 "endophytic fungi" memberi keputusan yang memuaskan dalam penskrinan bioaktif. Terdapat 16 pencilan mempamerkan keputusan positif dalam bio-aktiviti terhadap UCS/UCK. Ini menunjukkan 16

daripada 130 pencilan berpotensi menghasilkan kompaun bioaktif terhadap anti-tumor.

Empat daripada dua puluh dua pencilan dikenalpasti melalui pemeriksaan mikroskopik dan penjujukkan gen 18S/ITS. Pencilan 12L dikenalpasti sebagai *Penicillium* spp. melalui penelitian morfologi secara mikroskopik. Pencilan-pencilan yang dikenalpastikan dengan penjujukan 18S/ITS termasuk 1B, *Endothia gyrosa;* 19L, *Colletotrichum gloeosporioides* dan 22L, *Botryosphaeria ribis*. Program BLAST digunakan sebagai alat untuk mempastikan homologi jujukan yang diperolehi dengan database jujukan.

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LIST OF ABBREVIATIONS

%	Percent
µl	Microlitre (s)
^0C	Degree Celsius
BLAST	Basic Local Alignment Search Tool
bp	Base pair
cm	Centimetre (s)
CMA	Cornmeal Agar
CO_2	Carbon dioxide
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide Triphospate
e.g.	*Exempli gratia* (for example)
$FeSO_4$	Ferum Sulphate
g	Gram (s)
H_2O	Water
i.e.	*Id est* (that is)
IGS	Intergenic Spacer
ITS	Internal Transcribed Spacer
KCl	Potassium Chloride
KH_2PO_4	Dihydrogen Potassium Phosphate
LSU	Large Subunit
MEA	Malt Extract Agar
mg	Milligram (s)
mg/l	Milligram per Litre

$MgCl_2$	Magnesium Chloride
$MgSO_4$	Magnesium Sulphate
min.	Minute (s)
ml	Millilitre (s)
mM	Millimolar
MSP	Maximal Segment Pair
nd	None detected
ng	Nanogram (s)
NH_4NO_3	Ammonium Nitrate
O_2	Oxygen
PDA	Potato Dextrose Agar
RNA	Ribonucleic Acid
rpm	Rotation per minute
SSU	Small Subunit
Taq	*Thermus aquaticus*
TLC	Thin Layer Chromatography
U	Unit
UV	Ultra violet
v	Volume

CHAPTER I

INTRODUCTION

To microbes, land plants present a complex, spatially and temporally diverse ecological habitat. Symbiotic associations between microorganisms and plants are ancient and fundamental, and many examples of complex and highly specific symbioses between plants and microbes have been well known. Endophytic microbes are an intriguing group of organisms associated with various tissues and organs of terrestrial and some aquatic plants, and are the subject of increasing interest to mycologists, ecologists, and plant pathologist.

Biologically and ecologically, endophytes represent a diversity of nutritional modes from biotrophic parasites to interim or facultative saprotrophs, and associations with their hosts span the continuum from biotrophic mutualist and benign commensals to nectotrophic, antagonistic pathogens. Ecologically, interactions between host plants, pathogens, and herbivores are mediated by endophytes. The species composition and distribution of endophytic associations within and among hosts, and the reciprocal ecological effects of endophyte colonisation on host fitness and the composition of plant communities, are problems of common interest to endophytologists and plant ecologists. Research interests in endophytes are correspondingly diverse and include community analysis, anatomical-histological relationships, organismal biology, biodiversity studies, population

ecology and evolutionary biology, and ecological interactions among endophytes, hosts, and herbivores. Endophytism may be an important factor in microbial speciation and biodiversity. Accumulating evidence suggests that endophytes represent a large reservoir of genetic diversity and a rich source of heretofore undescribed species. Practical applications of endophytes include potential biological control agents, sources of novel metabolites for medicine, plant protection, and industrial use, and as research model systems for investigation of host-parasite interactions and evolution in natural systems.

Often the terms "endophyte" and "endophytic" are used with particular meaning by different workers and for particular groups of hosts and microbes. Among the definitions proposed for the term endophyte are "fungi colonising living plant tissue without causing any immediate, overt negative effects" (Hirsch and Braun, 1992). This definition includes virtually the entire spectrum of symbiotic interactions in which fungi and plants participate: parasitism, commensalism, and mutualism (Bills, 1996). This definition, however, fails to include prokaryotic microbes, such as bacteria and blue-green algae, or endophytic vascular plants (Fisher *et al.*, 1992; Mauseth *et al.,* 1985). A more inclusive definition of "endophyte" should stress the symptomless nature of the infection on the host without limiting the term to any particular group of organisms. Latent and quiescent pathogens are endophytes as are mutualistic microbes and benign commensals. Petrini (1991) considers the term endophyte to be purely topographical: "Endophytes colonise symptomlessly the living, internal tissues of their host,