BIOACTIVE COMPOUNDS FROM ENDOPHYTIC FUNGI OF THAI MEDICINAL PLANTS

WATTANA PANPHUT

A THESIS SUBMITTED IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR
THE DEGREE OF MASTER OF SCIENCE (MICROBIOLOGY)
FACULTY OF GRADUATE STUDIES
MAHIDOL UNIVERSITY
2001

ISBN 974-04-0900-8

๓๑๐

- 4 S.A. 2544

โครงการ BRT ชั้น 15 อาคารมหานครยิบซั่ม
539/2 ถนนศรีอยุธยา เขตราชเทวี กรุงเทพฯ 10400

BIOACTIVE COMPOUNDS FROM ENDOPHYTIC FUNGI OF THAI MEDICINAL PLANTS

WATTANA PANPHUT

A THESIS SUBMITTED IN PARTIAL FULFILLMENT

OF THE REQUIREMENTS FOR

THE DEGREE OF MASTER OF SCIENCE (MICROBIOLOGY)

FACULTY OF GRADUATE STUDIES

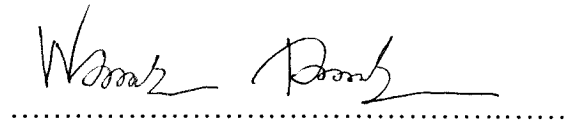
MAHIDOL UNIVERSITY

2001

ISBN 974-04-0900-8

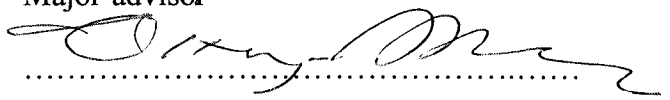
Thesis
entitled

BIOACTIVE COMPOUNDS FROM ENDOPHYTIC FUNGI OF THAI MEDICINAL PLANTS



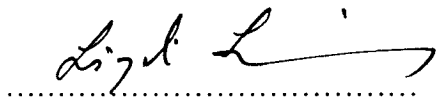
Mr. Wattana Panphut
Candidate

Asst. Prof. Suthep Wiyakrutta, Ph.D.
Major-advisor

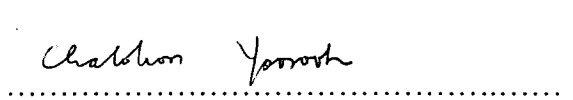


Prof. Vithaya Meevootisom, Ph.D.
Co-advisor

Assoc. Prof. Nongluksna Sriubolmas, Ph. D.
Co-advisor



Prof. Liangchai Limlomwongse
Ph.D.
Dean
Faculty of Graduate Studies



Prof. Chalobon Yoosook, Ph.D.
Chairperson
Master of Science Programme
in Microbiology
Faculty of Science

Thesis
entitled

BIOACTIVE COMPOUNDS FROM ENDOPHYTIC FUNGI OF THAI MEDICINAL PLANTS

was submitted to the Faculty of Graduate Studies, Mahidol University

for the degree of Master of Science (Microbiology)

on

19 October, 2001

.....

Mr. Wattana Panphut
Candidate

.....

Asst. Prof. Suthep Wiyakrutta, Ph.D.
Chairman

.....

Prof. Timothy W. Flegel, Ph.D.
Member

.....

Prof. Vithaya Meevootisom, Ph.D.
Member

.....

Assoc. Prof. Nongluksna Sriubolmas, Ph.D.
Member

.....

Prof. Amaret Bhumiratana, Ph.D.
Dean
Faculty of Science
Mahidol University

.....

Prof. Liangchai Limlomwongse, Ph.D.
Dean
Faculty of Graduate Studies
Mahidol University

ACKNOWLEDGEMENT

I would like to express my sincere gratitude and deep appreciation to Asst. Prof. Dr. Suthep Wiyakrutta, my Principal Supervisor and Assoc. Prof. Dr. Nongluksna Sriubolmas my Associate Supervisor for their invaluable guidance, consultation, comments, suggestions and constructive criticisms. They are always ready to help, led, and advise me in every way that would bring me success and great development.

I wish to express my sincere gratitude to my Associate Supervisor, Prof. Dr. Vithaya Meevootisom for his constructive comments, supervision with sincerity and good intention throughout this study. I am equally grateful to my member of the advisory committee Prof. Dr. Timothy W. Flegel for his extensive guidance, helpfulness, and his valuable suggestion and comment on this thesis. My special appreciation is expressed to Assoc. Prof. Dr. Nijsiri Ruangrungsi for his help on plant samples, Prof. Dr. Peerapan Tan-ariya for her help and suggestion on anti-malarial assay, Asst. Prof. Dr. Prasit Palittapongarnpimp for his help and suggestion on anti-TB assay. Special thanks are also given to, Dr. Jeerapan Worapong for her suggestions and assistance in knowledge of endophytic fungi. And also, my thankfulness to Ms. Duangnate Isarankul Na Adyudhaya, all kitchen's members in Department of Microbiology and all nice friends in Faculty of Science, Mahidol University for their kind helps and friendships during my study.

Finally, I am thankful to my family and especially my parents who have shown their great patience, moral support, and encouragement in every way possible to enable me to succeed in my education.

Wattana Panphut

4036442 SCMI/M : MAJOR : MICROBIOLOGY ; M.Sc.(MICROBIOLOGY)
KEY WORDS : ENDOPHYTIC FUNGI / BIOACTIVE COMPOUND

WATTANA PANPHUT : BIOACTIVE COMPOUNDS FROM ENDOPHYTIC FUNGI OF THAI MEDCINAL PLANTS. THESIS ADVISORS : SUTHEP WIYAKRUTTA, Ph.D., VITHAYA MEEVOOTISOM, Ph.D., NONGLUKSNA SRIUBOLMAS, Ph.D., 174 p. ISBN 974-04-0900-8

Endophytic fungi are fungi that colonize internally and symptomlessly within the living tissues of plant hosts. The relation between the fungal endophytes and the host plant can range from mutualistic-symbiosis and commensalism to borderline parasitism.

The aim of this research was to study biological activities of metabolites produced by endophytic fungi of Thai medicinal plants. Healthy leaves and limbs were collected from 35 species (26 families) of Thai medicinal plants collected from forest areas of three geographical regions of Thailand. Fungal endophytes were isolated from the surface-sterilized plant specimens placed on water agar by isolation from hyphal tips. A total of 123 fungi isolates were selected and cultured in Malt Czapek (MCz) broth and Yeast Extract Sucrose (YES) broth. Crude extracts were prepared separately from culture supernate and from mycelium. The crude extracts were assayed for biological activities. Most active metabolites were found in culture broth extracts of both media rather than in mycelium extracts. Antimicrobial activities against *Staphylococcus aureus*, *Enterococcus faecalis*, *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Candida albicans*, *Trichophyton mentagrophytes*, *Mycobacterium tuberculosis*, and *Plasmodium falciparum* were found at the frequencies of 35%, 33%, 76%, 6%, 2%, 15% 52% 27%, and 24%, of the total extracts respectively.

When cultured on common mycological media, most of the fungal endophytes did not develop any reproductive structures. However, they did produce characteristic morphology and each could be considered a distinctive fungus. Phylogenetic analysis of rDNA sequences of one selected endophytic fungus isolate helped to place it into a taxonomic group. This rDNA sequence was found to be novel when compared to known sequences at GenBank.

This study demonstrated that Thai plants accommodate many fungal endophytes most of which were capable of producing bioactive metabolites. Endophytic fungi of Thailand constitute a virtually untapped bioresource. More research on these potentially valuable fungi could yield novel bioactive compounds useful for medicinal and agricultural applications.

4036442 SCMI/M : สาขาวิชา : จุลชีววิทยา ; วท.ม. (จุลชีววิทยา)

วัฒนา พันธุ์พืช : สารออกฤทธิ์ทางชีวภาพจากราเอนโดไฟท์ของพืชสมุนไพรไทย (BOACTIVE COMPOUNDS FROM ENDOPHYTIC FUNGI OF THAI MEDCINAL PLANTS). คณะกรรมการควบคุมวิทยานิพนธ์ : สุเทพ ไวยครุฑธา, Ph.D., วิทยา มีวุฒิสม, Ph.D., นงลักษณ์ ศรีอุบลมาศ, Ph.D. 174 หน้า. ISBN 974-04-0900-8

ราเอนโดไฟท์เป็นราที่อาศัยอยู่กับเนื้อเยื่อของพืชที่มีชีวิตโดยแทรกตัวอยู่ระหว่างเซลล์พืช มีความสัมพันธ์กับพืชที่มันอาศัยอยู่แบบภาวะได้ประโยชน์ร่วมกันหรือแบบไม่เสียประโยชน์ (mutualic symbiosis) รวมไปจนถึงภาวะการได้ประโยชน์กึ่งปรสิต (borderline parasitic) ระหว่างพืชกับราที่แฝงตัวอยู่

งานวิจัยนี้ทำการศึกษาสารออกฤทธิ์ทางชีวภาพที่สร้างจากราเอนโดไฟท์ที่แยกจากพืชสมุนไพรไทย โดยเก็บพืชสมุนไพรไทยจากป่าธรรมชาติ 3 แหล่งจำนวน 35ชนิด (26 family) นำส่วนของกิ่งและใบที่ไม่มีรอยโรค มาคัดแยกราโดยนำตัวอย่างพืชมาผ่านกรรมวิธีปราศจากเชื้อที่ผิวนอกและเลี้ยงบน water agar จากนั้นใช้วิธี hyphal tipping แล้วคัดเลือกราเอนโดไฟท์จำนวน 123 ชนิดมาเลี้ยงในอาหารเหลว 2 ชนิดคือ Malt Czapek (MCz) และ Yeast Extract Sucrose (YES) จากนั้นทำการสกัดสารจากส่วนของน้ำหมัก และส่วนของเส้นใยรา แล้วนำมาทดสอบหาฤทธิ์ทางชีวภาพต่าง ๆ พบว่าสารสกัดที่ได้จากน้ำหมักมีฤทธิ์ทางชีวภาพดีกว่าส่วนที่ได้จากเส้นใย โดยพบจำนวนราเอนโดไฟท์ที่ให้ฤทธิ์ทางชีวภาพต่าง ๆ ดังนี้คือ ฤทธิ์ต้านเชื้อ *Staphylococcus aureus* 35% ฤทธิ์ต้าน *Enterococcus faecalis* 33% ฤทธิ์ต้าน *Bacillus subtilis* 76% ฤทธิ์ต้าน *Escherichia coli* 6% ฤทธิ์ต้าน *Pseudomonas aeruginosa* 2% ฤทธิ์ต้าน *Candida albicans* 15% ฤทธิ์ต้าน *Trichophyton mentagrophytes* 52% ฤทธิ์ต้าน *Mycobacterium tuberculosis* 27% และฤทธิ์ต้านเชื้อมาลาเรีย *Plasmodium falciparum* จำนวน 24%

เมื่อนำมาเพาะเลี้ยงบนอาหารเลี้ยงเชื้อราทั่วไปจำนวน 5 ชนิด พบว่าราเอนโดไฟท์ส่วนใหญ่ไม่สร้างสปอร์ทำให้จัดจำแนกได้ยาก อย่างไรก็ตามวิธีการที่ศึกษานี้สามารถบอกความแตกต่างราแต่ละสายพันธุ์ได้โดยอาศัยโคโลนีของราที่มีลักษณะจำเพาะ เมื่อคัดเลือกราจำนวน 1 ชนิดมาเป็นตัวอย่างในการจัดจำแนกโดยใช้ลำดับนิวคลีโอไทด์ ของยีนของอาร์เอนเอที่เป็นส่วนประกอบในไรโบโซม (rDNA) นำมา Blast เพื่อเปรียบเทียบกับ rDNA ของราชนิดอื่น ๆ ที่ได้จัดจำแนกไว้แล้วในข้อมูลของ GenBank พบว่าราที่นำมาเป็นตัวอย่างนี้เป็นราชนิดใหม่ที่มีลำดับนิวคลีโอไทด์ต่างไปจากข้อมูลที่มีใน GenBank

จากงานวิจัยครั้งนี้สามารถสรุปได้ว่าพืชสมุนไพรไทยเป็นแหล่งที่อาศัยของราเอนโดไฟท์จำนวนมากที่มีความสามารถในการสร้างสารออกฤทธิ์ทางชีวภาพ ซึ่งราเหล่านี้นับเป็นทรัพยากรทางชีวภาพที่สำคัญสิ่งที่ค้นพบในการวิจัยครั้งนี้เป็นข้อมูลในการศึกษาถึงศักยภาพของราเอนโดไฟท์ที่จะผลิตสารออกฤทธิ์ทางชีวภาพชนิดใหม่ ๆ อันจะเป็นประโยชน์ต่อวงการแพทย์ และการเกษตรต่อไป

CONTENTS

	Page
ACKNOWLEDGEMENT	iii
ABSTRACT (ENGLISH)	iv
ABSTRACT (THAI)	v
LIST OF TABLES	xii
LIST OF FIGURES	xiv
LIST OF ABBREVIATIONS	xvii
CHAPTER	
I INTRODUCTION	1
Objectives	3
II LITERATURE REVIEW	
1. The endophytic fungi	4
2. Collection and isolation strategies	8
3. Surface sterilization	9
4. Selective antibiotic, nutrients and culture manipulation	12
5. Study of secondary metabolites from endophytic fungi	14
6. Identification of fungi	29
6.1 Morphological identification	30
6.2 Molecular identification	36
6.2.1 Taxonomy and characterization of fungi by PCR	37
6.2.2 Ribosomal DNA gene studies	39

CONTENTS (Cont.)

	Page
8.2 Fungal metabolite extraction	53
9. Determination of antimicrobial activities	54
9.1 Test microorganisms	54
9.2 Standardization of inoculum	56
9.3 Preparation of bacterial inoculum	56
9.4 Preparation of fungal inoculum	57
9.5 Assay procedure	57
9.6 Reading of microplate assay	58
10. The microdilution assay of compounds against *Mycobacterium tuberculosis*	59
10.1 Preparation of mycobacteria inoculum	59
10.2 Preparation of control drugs	59
10.3 Tested metabolite preparations	60
10.4 Alamar blue (MABA) assay for anti-TB activity	60
11. Antimalarial activity testing	62
11.1 Malarial parasites	62
11.2 Malarial cultivation supply	62
11.2.1 Culture medium	62
11.2.2 Normal human serum	63
11.2.3 Non-infected erythrocytes	63

CONTENTS (Cont.)

	Page
11.3 *In vitro* cultivation of *P. falciparum* and maintenance of continuous culture	64
11.4 Synchronization of the cultures	65
11.5 Cryopreservation	66
11.5.1 Freezing and storing	66
11.5.2 Thawing	66
11.6 Determination of antimalarial activity of fungal endophyte metabolites	67
11.6.1 Tested metabolite preparation	67
11.6.2 *In vitro* antimalarial activity	67
12. Endophytic fungal DNA Extraction and PCR amplification	68
12.1 Mycelium preparation	68
12.2 DNA extraction	68
12.3 Polymerase chain reaction (PCR)	69
13. DNA sequencing and phylogenetic analyses	72
IV RESULTS	
1. Medicinal plant samples and endophytic fungi isolates	73
2. Characterization of endophytic fungi	84
3. Crude metabolites from endophytic fungi	95
4. Viable count of standardized inoculum	96

CONTENTS (Cont.)

		Page
5.	Antibacterial and antifungal activity by INT microdilution assay	97
6.	Anti-*M. tuberculosis* activities of crude extract of endophytic fungi cultures	101
7.	Biological activity of endophyte isolates for antimalarial activity	104
	7.1 Growth and development of *P. falciparum* from *in vitro* culture	104
	7.2 *In vitro* morphological changes of *P. falciparum* caused by crude extracts of endophytes	105
	7.3 Anti-malarial activities against *P. falciparum*	108
8.	Ribosomal DNA sequence of endophytic fungi	110
	8.1 The PCR product of ITS1, 5.8S, and ITS2 regions of ribosomal DNA	110
	8.2 Nucleotide sequence of partial 18S and 28S sequences and complete ITS1-5.8S-ITS2 sequence of isolate Pind 6	111
	8.3 Phylogenetic analyses	112
V	DISCUSSION	
1.	Occurrence and diversity of endophytic fungi in Thai plants	115
2.	Fungal endophyte isolation technique	116

CONTENTS (Cont.)

		Page
	3. Microdilution assay and bioactive metabolite screening strategies	116
	4. Biological activities of extracts from endophytic fungi	118
	5. Ribosomal DNA sequence of endophytic fungi	120
	6. Endophytic fungi may related to fungi in plant disease	120
VI	CONCLUSION	122
REFERENCES		123
APPENDIX		142
BIOGRAPHY		174

LIST OF TABLES

TABLE		Page
1	Selected examples of isolation procedures for endophytes from woody plants.	10
2	Endophytic fungi known to produce taxol.	18
3	Biological activities of preussomerins 1-6 (structure in Figure 7) against microbial test by agar diffusion assay.	26
4	Minimum inhibitory concentrations of cryptocin for representative group of plant pathogenic fungi.	27
5	Primers for amplification of fungal ribosomal RNA genes.	41
6	Test microorganisms for standardization and microdilution assay.	55
7	Primers for amplification of fungal (Pind 6) ribosomal RNA genes.	71
8	List of Thai medicinal plants used in this study.	73
9	Selected isolates of endophytic fungi obtained from Thai medicinal plant samples.	77
10	The crude metabolites extracted from endophytic fungi.	94
11	Colony forming unit (CFU)/ml of standardized inoculum with turbidity matched to 0.5 McFarland standard (OD_{625} = 0.1).	96
12	Anti TB (*M. tuberculosis*) activities as determined by Alamar-blue microdilution plate assay (MABA).	103
13	Summary of biological activities of endophytic fungi.	119

LIST OF TABLES (Cont.)

TABLE		Page
14	Raw data for antibacterial and antifungal activity from culture broth extract of MCz medium	148
15	Raw data for antibacterial and antifungal activity from culture broth extract of YES medium	151
16	Raw data for antibacterial and antifungal activity from mycelium extract of MCz medium	154
17	Raw data for antibacterial and antifungal activity from mycelium extract of YES medium	157
18	Raw data for antimycobacterium activity of culture broth extract from MCz and YES medium.	160
19	Raw data for antimycobacterium activity of mycelium extract from MCz and YES medium.	164
20	Raw data for antimalarial activity of culture broth extracts from MCz and YES medium.	167
21	Raw data for antimalarial activity of mycelium extracts from MCz and YES medium.	170

LIST OF FIGURES

Figure		Page
1	The five interactive components that can be involved in a symptomatic plant disease	5
2	Structure of taxol (paclitaxel), a novel drug anticancer agent.	17
3	Taxol producing endophytic fungi isolated from *Taxus* spp.	19
4	Taxol producing endophytic fungi isolated from *Taxus* spp.	20
5	Alkaloid metabolites, loliterm B and peramine	23
6	*Echinopagon ovates*, the endophyte of perennial ryegrass	24
7	Preussomerin and its derivatives structure	25
8	The structure formula of cryptocin	27
9	Sporulation of endophytic fungi on different types of plant tissue in agar culture	34
10	Location on nuclear and mitochondrial rDNA of primers given in Table 5	40
11	11A; The Alamar blue fluorescent emission spectra and 11B; Alamar blue absorbance	61
12	Colonial morphology of an endophytic fungus isolate, Cinc 3	85
13	Colonial morphology of an endophytic fungus isolate, Dfil 8	86
14	Colonial morphology of an endophytic fungus isolate, Ijav 4	87
15	Colonial morphology of an endophytic fungus isolate Mcor 5	88

LIST OF FIGURES (Cont.)

Figure		Page
16	Colonial morphology of an endophytic fungus isolate, Mmal 1	89
17	Colonial morphology of an endophytic fungus isolate, Pind 6	90
18	Colonial morphology of an endophytic fungus isolate, Sgri 1	91
19	Colonial morphology of an endophytic fungus isolate, Tpal 1	92
20	Colonial morphology of an endophytic fungus isolate, Vden 3	93
21	Colonial morphology of an endophytic fungus isolate, Wtom 4	94
22	INT microdilution assay of anti-*S. aureus* activity (21A) and anti-*C. albicans* activity (21B)	99
23	A summary of INT microdilution assay results for antibacterial activity and antifungal activity by microdilution assay	100
24	Example of an Alamar blue microdilution method for anti-TB	102
25	Growth and development of *P. falciparum* from *in vitro* culture	106
26	*In vitro* morphological changes of *P. falciparum* as the result of exposure to crude extracts from endophytes	107
27	Anti-malarial activities of fungal endophyte crude extract	109
28	Agarose gel electrophoresis analysis of the PCR product from amplification of ITS1, 5.8S and ITS2 regions of rDNA	110
29	Nucleotide sequence of the ITS1, 5.8S and ITS2 regions of rDNA of isolate Pind 6	111

LIST OF FIGURES (Cont.)

Figure		Page
30	Consensus parsimonous tree inferred from the analysis of ITS1 sequence of isolate Pind 6	113
31	Consensus parsimonous tree inferred from the analysis of ITS2 sequence of isolate Pind 6	114

LIST OF ABBREVIATIONS

e.g.	for example
ITS	Internal transcribed spacer
5.8S	Small 5.8 subunit
28S	Large 28 subunit
DNA	Deoxyribonucleic acid
rDNA	Ribosomal deoxyribonucleic acid
%	Percent
&	And
spp.	Species
YES	Yeast Extract Sucrose medium
MEA	Malt Extract Agar
MCz	Malt Czapek medium
TWA	Tap Water Agar
etc.	for et cetera (Latin)
FDA	Food and Drug Administration
L^{-1}	per liter
µm	Micro meter
ELISA	Enzyme-Linked Immunosorbent Assay
et al.	and other (Latin)
mg	Milligram
ml	Milliliter
AM	Abuscular mycorrhizal

LIST OF ABBREVIATIONS (Cont.)

IAA	Indole 3-acetic acid
i.e.	meaning of (Latin)
μg	Microgram
mm	Millimeter
μl	Microliter
PCR	Polymerase Chain Reaction
RAPD	Random amplification of polymorphic DNA
AFLP	Amplified fragment length polymorphism
IGS	Intergenic spacer
SSU	small subunit
rRNA	Ribosomal ribonucleic acid
T_m	Melting temperature
bp	Base pairs
ETS	Externally transcribed spacer
NTS	Non-transcribed spacer
18S	18 small subunit ribosomal
ATCC	American Type Culture Collection
DMSO	Dimethyl sulfoxide
g	Gram
h	Hour
M	Molar
mM	Millimolar

LIST OF ABBRIVIATIONS (Cont.)

°C	Degree celsius
NTP	Nucleotide triphosphate
rpm	Round per minute
TBE	Tris-Borate EDTA
TE	Tris-EDTA
U	Unit
V	Volt
v	Volume
w	Weight
OD	Optical density
CMA	Corn meal agar
PDA	Potato dextrose agar
SDA	Sabouraud dextrose agar
TB	*Mycobacterium tuberculosis*
INT	*p*-iodonitrotetrazolium violet
CFU	Colony forming unit
MHB	Mueller-Hinton broth
MIC	Minimal inhibition concentration
LC	Lethal concentration
RBCs	Red blood cells

CHAPTER I

INTRODUCTION

Health problems are still a serious topic in Thailand. These problems include a drug resistance in malarial infection, tuberculosis emerging on AIDS patients and also common infectious diseases. Increased efforts are therefore needed to develop and search for new drugs from natural bioresources. Of the drug-producing microbes employed for pharmaceutical industry, fungi are probably not only the most important, but also the most poorly studied organisms. They play an integral role in ecosystem processes, *e.g.* nutrient cycling, plant growth, and animal nutrition. Because of their sensitivity to air pollution they are sometime used as air quality indicators. They also have a huge potential in the agricultural, pharmaceutical and health care industries. Currently, approximately 72,000 species of fungi have been described, and Hawksworth (1) conservatively estimated that there are 1.5 million fungal species. Fungi form a wide variety of interactions with plants, from benign forms of mutualism through to devastating crop diseases. Although plant diseases are well known and of continuing economic importance, the fact that plants seldom if ever, exist without fungal infection either endophytic or mycorrhizal type is perhaps not so widely appreciated.

Endophytic fungi are microorganisms that commonly live in intercellular spaces of living plant hosts. They may provide their hosts with metabolites and other potentially useful bioactive compounds. The association of these microorganisms with

higher plants ranges from mutualistic symbiosis or commensalism to borderline latent pathogenicity (2). Endophytes can be transmitted from one generation to next through the tissue of host seeds or vegetative propagules. Some grass endophytes appear to be transmitted horizontally, external to host tissues, with their aerial spores.

According to Dreyfuss and Chapela (3), approximately 4,000 metabolites, of fungal origin have been described to possess biological activities. It has been estimated that there are 270,000 global vascular plant species (4), with tropical regions having the most diverse variety and more than half the total number worldwide (1). Thailand located in a tropical rainforest region with many enrich and varieties of plants that may support endophytes. Endophytic fungi are probably an important resource for novel metabolites with pronounced antibacterial, antifungal, antiviral, and anticancer activity. For example, many endophyte species have been found to produce the novel drug, taxol and other new compounds. The present research comprised a search for bioactive compounds produced by endophytic fungi isolated from Thai medicinal plants.

Objectives

1. To isolate endophytic fungi from Thai medicinal plants.
2. To determine biological activities of the extracts from endophytic fungal cultures, including antibacterial, antifungal, antimycobacterial and antimalarial activities.
3. To study macroscopic morphology of endophytic fungi grown on different culture media.
4. To sequence the ITS1, 5.8S, and ITS2 of rDNA of a selected endophytic fungus isolate and to perform a phylogenetic analysis for its taxonomic classification.

CHAPTER II

LITERATURE REVIEW

1. Endophytic Fungi

The past 15 years has seen a proliferation of investigations that are only beginning to uncover the vast internal mycota of living plant tissue and its functions. For the purpose of this discussion, a broad definition of endophytism is used, that being fungi established inside healthy plant tissue without causing overt symptoms in or apparent injury to the host (5, 6). The term "endophyte", whose common usage has evolved only in recent times, is now accepted as a useful name for categorizing a myriad of plant associated fungi familiar to those engaged in diagnosis of plant diseases and identification of plant-inhabiting fungi. Endophytic fungi form inconspicuous infections within tissues of healthy plants for all or nearly all their life cycle (7). Endophytes, in contrast to epiphytes, are contained entirely within the substrate plant and may be either parasitic or symbiotic. Endophytic fungi are asymptomatic and may be described as mutualistic (8). The major features of mutualistic symbiosis include the lack of destruction of most cells or tissue, nutrient or chemical cycling between the fungi and their host. There were enhanced longevity and photosynthetic capacity of cells and tissues under the influence of infection, enhanced survival of the fungus, and a tendency toward greater host specificity than seen in nectrophic infection (9). A comparison of the fitness of the host and fungus when living

independently determining whether a specific symbiotic association is mutualistic or parasitic (10). Members of the Ascomycotina, Basidiomycotina, Deuteromycotina, and some Oomycetes have been isolated as endophytes. Endophytic fungi have been isolated from phanerogams (seed plants) in alpine, temperate and tropical regions, although the plants of the Coniferae, Ericaceae, and Gramineae have been most intensively sampled (7, 8, 11).

By definition, endophytic colonization or infection cannot be considered as causing disease, since a plant disease is an interaction between the host, parasite, vector and the environment over time (Figure 1), which results in the production of disease signs and/or symptoms (12).

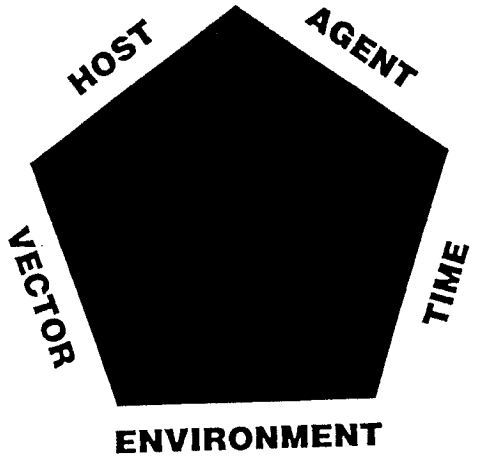


Figure 1. The five interactive components that can be involved in a symptomatic plant disease (12).

The distinction between an endophyte and pathogen is not always clear. A mutation at a single genetic locus can change a pathogen to a nonpathogenic endophytic organism with no effect on host specificity (13). Many pathogens undergo an extensive phase of asymptomatic growth corresponding to colonization and then latent infection before symptoms appear. Many pathogens of economically important crops may be endophytic or latent in weeds (14, 15, 16). Alternately, nonpathogenic endophytic organisms may play a role as biocontrol agents (3). Both endophytic and latent infection fungi can infect plant tissues and become established after penetration, however, infection does not imply the production of visible disease symptoms.

Long before the recent interest in endophytic fungal communities, basic methods for study of endophytes had been well established by those studying fungal disease of woody plants and interactions among fungal pathogens and other fungi resident within the same host. What differs about ongoing investigations of endophytic fungi, is that single causal organisms of disease have not been pursued, but all plant tissues have been surveyed on a systematic basis for culturable internal fungi in a manner analogous to ecological investigations of vegetation landscapes. During studies of various host plants, the internal fungi have been sampled in very methodical patterns with the goal of describing the spatial distributions and temporal sequences of these fungal assemblages. Investigators have tried to ascertain what are the underlying anatomical features, developmental attributes, host preferences, environmental and climatic cues, and geographical factors that determine colonization strategies of endophytic fungi (6). Because of the inherent obstacles to direct observation and identification of fungi inside living plants, probing

endophytic fungal communities has depended heavily on observation and correlative data complied from exhaustive isolations from plant tissue. As always, the interpretation of microbial community structure based on isolation organisms produces biased results related to the selective action of the isolation methods and sampling patterns.

Most procedures for isolating endophytes are comparatively simple and routine for one skilled in basic plant pathological or microbiological technique. However, the process of designing an analytical study of an endophyte community, handing and maintaining the often hundreds of isolates, characterizing the isolates taxonomically, and quantitatively interpreting the results can be burdensome and overwhelming. The techniques and materials used for isolation, maintenance, identification and preservation of endophytes of grasses were reviewed recently (17). Grass endophytes are an extremely host-specific and specialized subset of endophytes with their own peculiar life cycles. The isolation and identification methods for endophytes of non-grass phanerograms (seed plants) was last reviewed by Petrini (11) in which he presented a useful table listing surface sterilization protocols for various kinds of plants and plant organs.

Since that time, dozen of articles have appeared that describe the isolations and analyses of fungal communities inhabiting woody plants. A staggering accumulation of data is now available that correlates the occurrence of fungi within organs of various plant taxa (18). A discussion of various isolation techniques, sampling strategies and identification methods for endophytes of leaves, stems, and root of woody plants are presented in the following section of this thesis.

2. Collection and Isolation Strategies

The needs of the investigator will necessarily dictate which approach will be followed for plant selection and isolation procedures for endophytes. The logistics of moving fresh plant materials while maintaining plant tissues in good condition can be a formidable job when working with plants from remote areas. Precautions must be taken in the shipment of living plant material for culture isolation purposes so the need to prevent desiccation and tissue death is balanced with the requirement for proper ventilation. Aeration maintains respiration, prevents overhumid conditions and suppresses growth of epiphytic fungi and bacteria. Therefore, prolonged transport in sealed plastic bags should be avoided if possible. Avoiding overhumid storage conditions and elevated temperatures is nearly impossible when collecting plants in remote humid tropical forests. When plants are collected under wet tropical conditions, surfaces should be thoroughly air-dried prior to packing and shipping. Bernstein and Carroll (19) recommended transporting conifer foliage in unclosed bags for local transport. Baird (20) maintained spatial position and orientation of bark plugs between the field and laboratory by ordering the plugs in precoded sterile microtiter plates. However, long distance shipping of tropical plants in polyethylene bags has yielded acceptable results (21, 22). If plants are stored for long periods, especially in frost-free refrigerators, tissue desiccation will occur with a subsequent loss of some internal fungal species. Still, a surprising number of species can be isolated from desiccated woody tissues even after freezer storage of more than a year (Bills & Poilhook, unpublished) (23).

3. Surface Sterilization

Probably no other step is as critical to obtaining good results as thorough but non-penetrating surface sterilization. The possibility that isolates have been initiated from propagules on the surface must be minimized. The choice of sterilization times, concentrations, and volumes will be dictated by the thickness of its surface (Table. 1). Selection of isolates that have emerged from the cut ends of surface sterilized grass leaves has been practiced to ensure that only internal fungi were selected (24). Such strict criteria usually have not been applied to isolations from woody plants.

Sterilization methods continue to vary widely, but the preferred method is a three-step ethanol, sodium hypochlorite (NaOCl), and ethanol treatment (11). Independent tests demonstrated that sequential treatment with ethanol-NaOCl-ethanol effectively killed the thick-walled ascospores of *Sporormiella intermedia* (Auersw.) Ahmed & Cain (25), and conidia of *Heliscus lugdunensis* (Zins) Scholten (26). It should be noted that sodium hypochlorite solutions spontaneously decompose in storage and therefore percentages calculated from labels of household bleach should not be considered accurate. Hydrogen peroxide solutions, commonly used as disinfectants, have not been widely used in endophyte isolation studies. Surface sterilization with 0.05 to 1 % peracetic acid in 30 % ethanol may be a more accurate alternative to sodium hypochlorite solutions (M.M. Dreyfuss, personal communication). Ethanol (96 %) followed by mercuric chloride (0.1 %) and sterlie water was used to surface sterilize leaves of *Eucalyptus viminalis* Labill. (23). Leaf surface of *Populus tremuloides* Michx. were disinfected by silver nitrate (0.1 %) followed by sodium chloride rinses (28).

Table 1. Selected examples of isolation procedures for endophytes from woody plants (23).

Tissues/hosts (reference)	dissection	surface sterilization	media	comments
spines and stems of *Ulex* spp. (50)	spines and 2 cm stem sections	1 min 96% ethanol, 3 min 3.25% NaOCl, 0.5 min 96% ethanol	malt extract agar	frequency of colonization increased from new to 2 yr old stems
bark of *Fagus grandifolia*, (37)	2 mm borer	propane torch or none	bark extract, glucose - yeast extract, benomyl malt extract	bark plated in the field
xylem and bark of *Alnus* spp. (53)	1 cm stem sections, bark and xylem cultured separately	35% peracetic acid	malt extract with 10 mg/l cyclosporin A	multivariate analysis revealed bark and xylem and the 3 different *Alnus* spp. can be distinguished on the basis of fungal communities
bark of *Castanea dentata, Quercus rubra* (7)	5 mm arch punch	0. 525% NaOCl for 10 minutes	glucose-yeast extract medium	microtiter plates for maintaining sequence of bark samples
mature stems of *Picea abies* (110)	sawing followed by chisel extraction	only extraction tools sterilized	malt agar in slants	frequency of isolates were enumerated along transverse and longitudinal axes of sound and wounded wood
roots of *Picea abies* (68)	1 cm segments	sink washing, ultrasound, serial washing	malt extract agar	3 primary fungal assemblages identified that were correlated with site altitude and edaphic factors
twigs of *Fraxinus excelsior, Quercus robur, Fagus sylvatica* (64)	2 cm segments, bark and xylem cultured separately	ethanol flaming	malt extract agar	succession of decomposition followed after stem girdling
bark of *Carpinus caroliniana* (16)	1 cm leather punch	0. 525% NaOCl for 3 minutes, flaming in alcohol lamp	malt yeast extract agar with or without benomyl and surfactants, Mycosel agar	imperfect surface sterilization compared with sequential 3-step procedure, use of quartered Petri dishes
seedlings of *Rhizophora mangle* (87)	1.1-2 cm cork borer of hypocotyl and radicle	sterile sea-water rinse, 0.1% HgCl2 in 5% ethanol	mangrove-sea water agar, 4% mangrove ground mangrove seedling in sea water	succession of mycoflora followed in seedlings maintained in nylon mesh bags
leaves of *Licuala ramsayi* (108)	3 mm discs from veins and interveins	1 min 96% ethanol, 10 min 3.25% NaOCl, 0.5 min 96% ethanol	commeal dextrose agar, malt extract agar	leaves shipped in muslin bags, higher frequency of endophytes in veins, endophytes in veins of unopened leaves
leaves of *Euterpe oleracea* (109)	3 mm discs from veins and interveins	1 min 75% ethanol, 10 min 3.25% NaOCl, 0.5 min 75% ethanol	commeal dextrose agar with 5 mg/l cyclosporin A	leaves processed within 24 hr of collecting

Ethanol immersion followed by flaming can also yield reasonably good results with larger diameter twigs, limbs and wood fragments. Stems can be dipped in ethanol and ignited with a Bunsen burner or alcohol lamp (29, 30) and whole stem pieces can be used or the bark and underlying xylem are cultured separately. Flaming bark of *Fagus grandifolia* in the field with a propane torch before chip extraction reduced the isolation frequency of moniliaceous hyphomycetes (e.g. *Penicillium* spp., *Fusarium* spp., *Cladosporium* spp.) and algae (31).

Serial washing offers the advantage of eliminating the penetrating and killing effects of sterilizing chemicals. Prewashing with tap water can help reduced the time needed for surface sterilization. This is especially important if very tiny fragments of tissues are being used. Washing also may be used in conjunction with ultrasonic cleaning and/or detergents. However, with washing alone, in all likelihood, some colonies will arise from propagules embedded in surface irregularities.

A serial washing procedure based on the classic soil-washing technique (32) was used to phylloplane fungi and epiphytes from pine needles (33). When compared to untreated leaf surfaces, serially washed and surface-sterilized leaf discs both yielded comparable results in terms of frequency of species isolated. Both kinds of surface treatments demonstrated that leaves were colonized by a distinctive group of terminal parasites and saprobes while still living on the tree and that many of these species were actively colonizing senescent and dead leaves before being incorporated into the litter.

4. Selective Antibiotics, Nutrients and Culture Manipulation

The phytopathological literature abounds with media formulations designed to recover targeted pathogens from various substrates (34, 35). These kinds of media may be useful if the goal is an autoecological study of a single endophytic species. Frequently however, the primary objectives in studying endophytic communities require media and isolation procedures that recover the broadest range of internal fungi, while discouraging or eliminating interference by external fungi. Because several media that are nutritionally enriched enough to support slow-growing fungi invite inundation by rapidly growing and heavily sporulating fungi, it then becomes necessary to eliminate or restrict the latter by physical or chemical means. Thus, to achieve the isolation of the total culturable fungal community, destructive chemical and physical procedures must be in equilibrium with the need to initiate colonies from vegetative structures inhabiting internal tissues. Incorporation into agar media of sublethal doses of fungitoxic agents that restrict radial growth of colonies, either singly or in combination, can effectively suppress rapid-growing epiphytic fungi, such as *Trichoderma*, *Pencillium*, *Alternaria*, or *Cladosporium* spp. Similarly radial growth rates range widely among different endophytes. Fast-growing endophytes such as *Nodulisporium, Phomopsis, Pestalotiopsis, Botryosphaeria* or *Furarium* spp. will often overwhelm plates. Antibacterial antibiotics should always be included in any primary isolation medium of fungi. Oxytetracycline, chlortetracycline, streptomycin sulfate, and novobiocin have been used most frequently for endophyte isolation. Chloramphenicol is an especially easy to use antibacterial agent because it is relatively heat stable and withstands autoclaving (36).

Diverse media have been used for the cultivation of filamentous fungi. For biochemical and physiological work it is necessary in most cases to use media which are chemically defined. It is recognized generally that liquid media are preferred for physiological studies. However, for rapid screening of numerous strains to provide taxonomic characters, agar media has proven to be more practical.

Culture media used for cultivation of fungi show a marked effect on fungal metabolite production. Czapek yeast autolysate and yeast extract sucrose are mainly used for production of intracellular and extracellular metabolites, respectively (38). Many of general basal media for secondary metabolite production contain high concentrations of carbon source, usually as sucrose, and organic extracts to provide a wide range of growth factors, for example Yeast Extract Sucrose (YES) (38). For the production of secondary metabolites, Malt Extract Agar (MEA), and Malt Czapek Agar (MCz) were suggested by Smith (39). Organic extracts are undefined media containing metabolites derived from plants, animal, or microbial sources. Commonly used natural substances include malt extract, yeast extract, and cornmeal. These natural materials promote favorable growth of fungi because they contain an array of sugars, proteins, vitamins, and minerals that can be readily assimilated. Such natural media are valuable for the routine culture of many fungi because they are inexpensive and simple to prepare and provide excellent growth of many fungi (40).

One further agar based medium that is useful for the isolation of fungi is Tap Water Agar (TWA) (41). This medium (TWA) is advantage for primary endophyte screening. It is a minimal medium, which ensures that fungi can grow slowly and hyphal tips can be

easily isolated because the fungi get nutrients only from the plant segments without basal supplement from the medium.

5. Study of Secondary Metabolites from Endophytic Fungi

Microorganisms, in particular fungi, are important sources of novel secondary metabolites. Secondary metabolites are those that are not essential for vegetative growth. Their production often occurs as growth rate declines and during the stationary phase. Some are associated with the differentiation and sporulation that may accompany the stationary phase. Fungal secondary metabolites are a diverse group of compounds produced by a wide range of different fungi, normally after a phase of balanced growth (40, 41, 42). Since the formation of secondary metabolites is associated with the stationary rather than the growth phase, batch rather than continuous culture is usually favored for their production. Secondary metabolites made by several fungi *e.g.* penicillin, lovastatin and cyclosporin, are of great economic importance in the pharmaceutical industry. Others are microbial agro-active compounds, useful as insecticides, herbicides of fungicides: milbemycins, bialaphos and kasugamicin are typical examples. Sophisticated screening and process methodology has yielded a wide range of novel bioactive molecules, with enzyme-inhibitory, immunomodulatory, antitumor, anticoccidial or antiprotozoal activities, as well as unusual enzymes, bioflavours, vitamins, pigments, specialty sugars and other fine chemicals (42, 43). The number of fungal species in the world was estimated to be at least 1.5 million by Hawksworth in 1991 (1). Thus, fungi can potentially be the major sources for new and useful metabolites.

According to Dreyfuss and Chapela (44), approximately 4,000 secondary metabolites of fungal origin have been described to possess biological activities. The largest part of these metabolites is produced by a group of fungi that Dreyfuss and Chapela called "creative": they include, among other, species of *Acremonium*, *Aspergillus*, *Fusarium* and *Penicillium*.

Endophytic fungi associated with higher plants range from mutualistic-symbiotic and commensal to borderline latent pathogenic. Mutualism between plants and endophytes occurs when former protects and feeds the latter which produces in return bioactive (plant growth regulatory, antibacterial, antifungal, antiviral, insecticidal, *etc.*) substances to enhance the growth and competitiveness of the host in nature (12). There are perhaps as many as 500,000 distinct varieties of higher plants. The varieties of fungi associated with such plants are estimated to be at least as high, suggesting that the extent of this biodiversity is enormous (2). As tropical regions host more than half the number of living species worldwide, a large number of biologically active metabolites is probably produced in these ecosystems. This is also supported by the fact that several plant species of the tropics are known to possess medicinal properties. Endophytic fungi represent a pharmaceutically valuable but virtually untapped resource. Few of the world's fungi, approximately 5 % of worldwide species have been identified. The rest, including a huge group of plant endophytes, are not yet explored (2). Research on fungal endophytes is limited, much of it focusing on identifying several species associated with conifers.

Endophytes from the Yew tree were primitively used as models for studying the biological interaction of bioactive compounds between host plant and fungi. The bark of

the Pacific yew (*Taxus brevifolia*) was discovered to be a source of an anticancer drug taxol (paclitaxel) in 1971 by The National Cancer Institute (NCI) (45). The chemical structure of taxol is shown in Figure 2 (46). Taxol was a novel compound whose activity was clinically shown to be active against breast and ovarian cancers. This drug appeared to block depolymerization of microtubule formation, resulting in the propagation of cancer cells. Its blocking mechanism was unique when compared to other anticancer drugs previously known (46, 47, 48, 49). The Food and Drug Administration (FDA) approved taxol as a new anticancer drug in 1993 (48). Although it could be completely synthesized by chemists, this was complicated, requiring more than 30 synthesis steps resulting in a low yield of only 0.05 %. This is not commercially available (50). To date, taxol on the world's market is wholly or partially derived from yew trees (51).

In 1993, Strobel *et al.* firstly announced that taxol was produced by an endophytic fungus *Taxomyces andreanae* that was found growing on one particular specimen of yew tree (*T. brevifolia*) (51). Taxus is a rich source of endophytic fungi that obtained from Glacier National Park, Montana, it yielded the fungus *T. andreanae.* Later Strobel's group reported another endophyte taxol-producing fungus, *Pestalotiopsis microspora*, which was obtained from a non-yew source (52). The taxol-producing endophytic fungi were shown in Table 2 (51). Each isolate was identified after sporulation on gamma-irradiated carnation leaves supported on water agar (51). This helped to further characterize taxonomic aspects including spore morphology, color, and method of sporulation (Figure 3 and 4) (51).

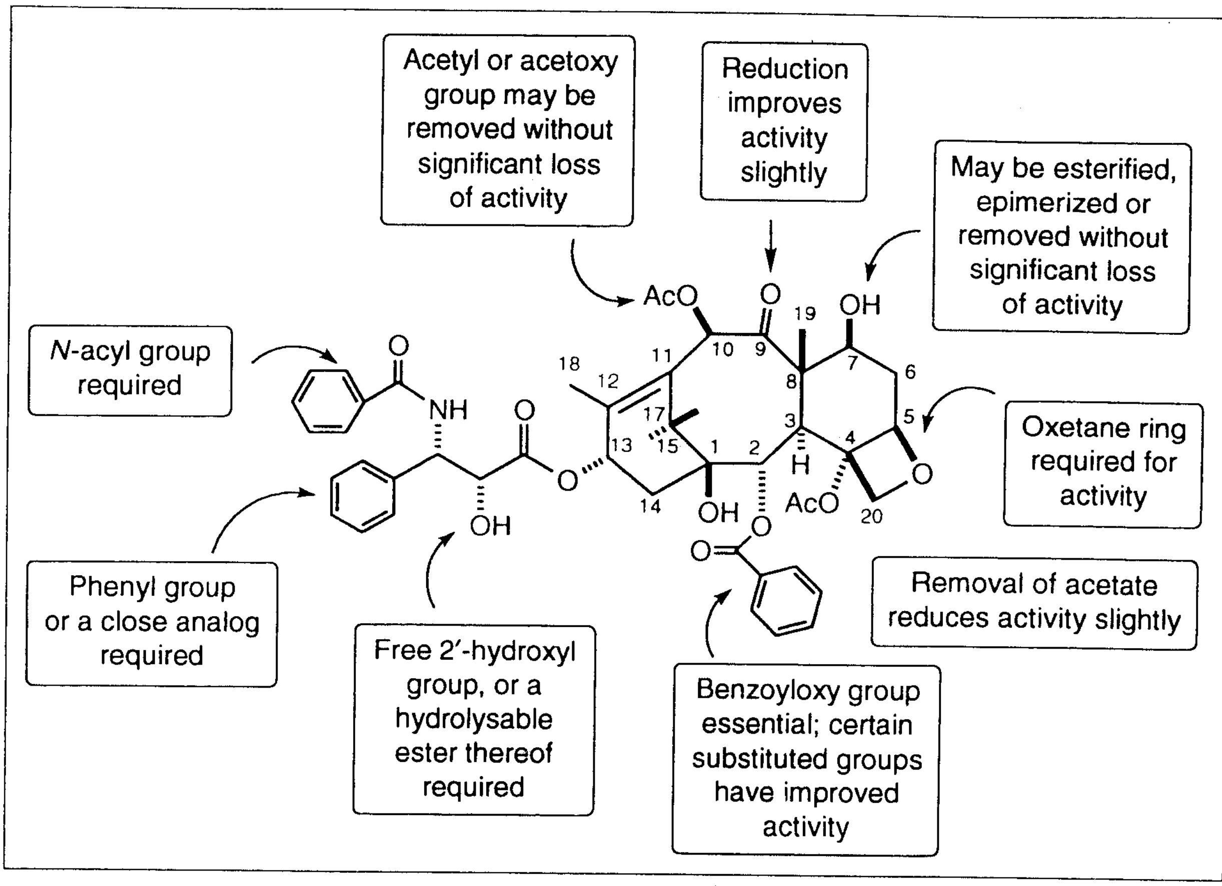
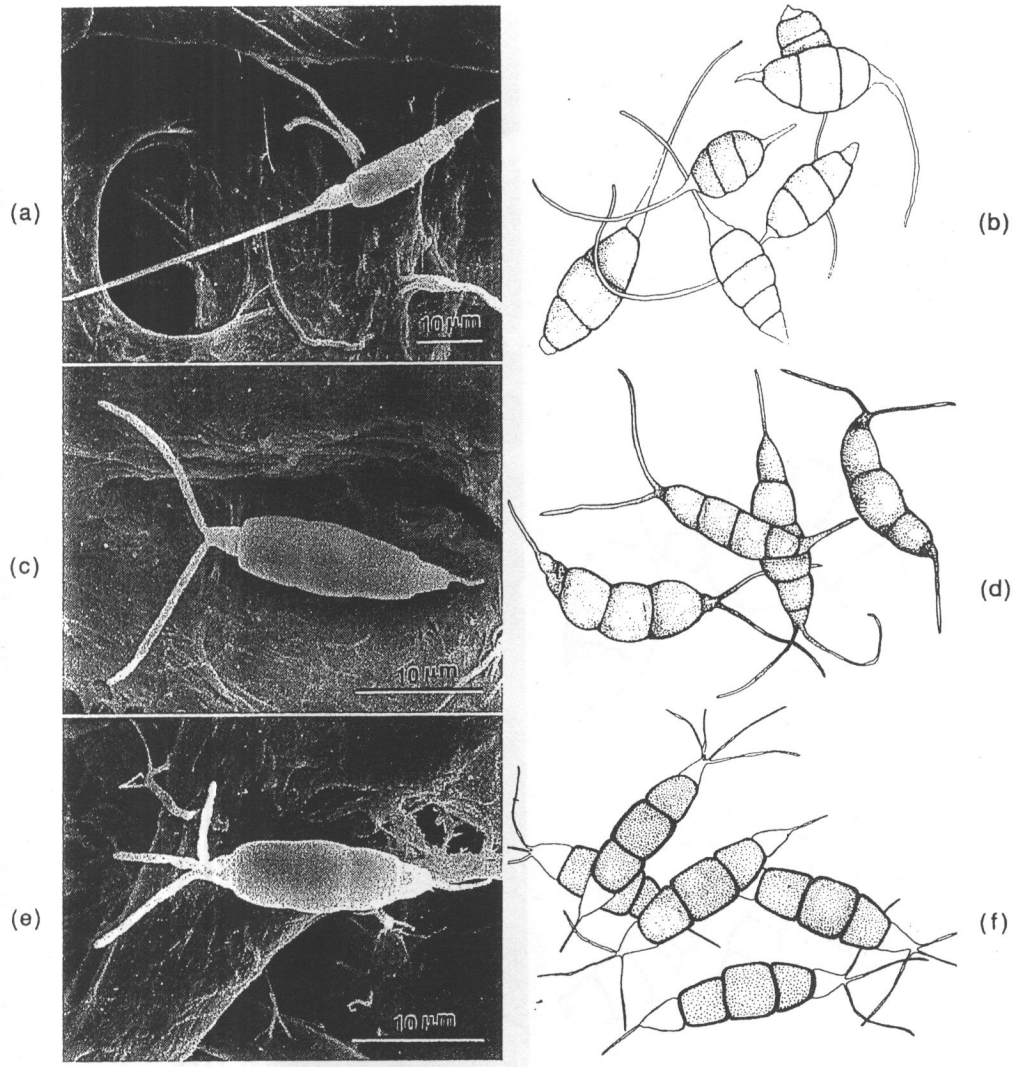


Figure 2. Structure of taxol (paclitaxel), a novel drug anticancer agent. The structure-active relationships between the functional groups of taxol are also presented. (46).

Table 2. Endophytic fungi known to produce taxol (51).

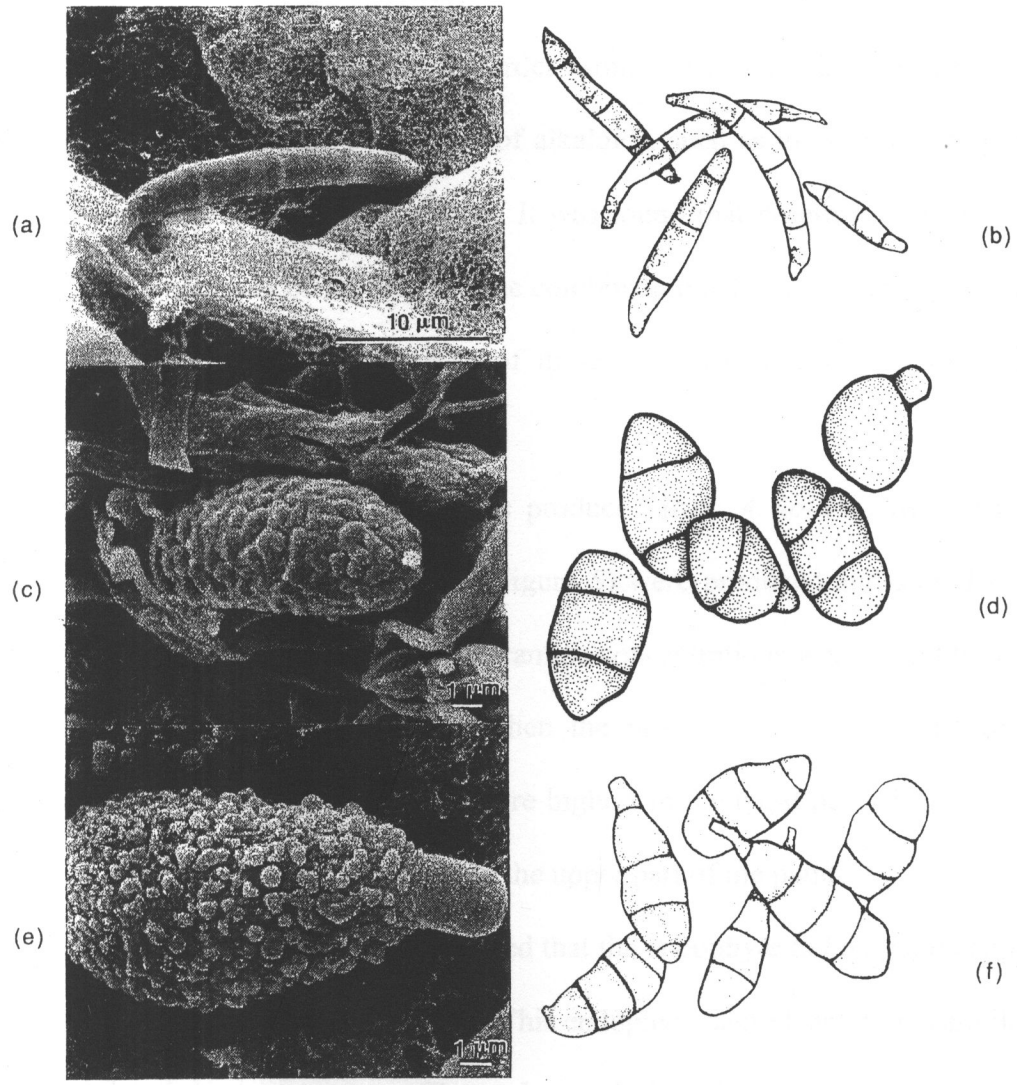
Culture reference No.	Taxus source	Fungus	Taxol L^{-1} [a]
Tbp-2	*T. baccata*	*Monochaetia* sp	102
Tbp-9	*T. baccata*	*Fusarium lateritium*	130
Ja-69	*T. cuspidata*	*Alternaria* sp	157
Ja-73	*T. cuspidata*	*Pestalotiopsis microspora*	268
Ne-32	*T. wallachiana*	*Pestalotiopsis microspora*	500
P-96	*T. sumatrana*	*Pithomyces* sp	95
Tbx-2[b]	*T. baccata*	*Pestalotia bicilia*	1081

[a]Taxol measured quantitatively using monoclonal antibodies.
[b]This fungus was obtained from the stem—xylem, while all others were from the phloem/cambium.



(a) and (b) *Monochaetia* spp isolated from *T. baccata*. (a) Scanning electron micrograph of a spore on filter paper. (b) Drawing of spores. (c) and (d) *Pestalotia bicilia* isolated from *T. baccata*. (c) Scanning electron micrograph of a spore on filter paper. (d) Drawing of spores. (e) and (f) *Pestalotiopsis microspora* isolated from *T. cuspidata* and *T. wallachiana*. (e) Scanning electron micrograph of a spore on filter paper. (f) Drawing of spores.

Figure 3. Taxol producing endophytic fungi isolated from *Taxus* spp. (51).



(a) and (b) *Fusarium lateritium* isolated from *T. baccata*. (a) Scanning electron micrograph of a spore on a leaf surface. (b) Drawing of spores. (c) and (d) *Pithomyces* sp isolated from *T. sumatrana*. (c) Scanning electron micrograph of a spore on a leaf surface. (d) Drawing of spores. (e) and (f) *Alternaria* sp isolated from *T. cuspidata*. (e) Scanning electron micrograph of a spore on a leaf surface. (f) Drawing of spores.

Figure 4. Taxol producing endophytic fungi isolated from *Taxus* spp. (51).

Perennial ryegrass (*Lolium perenne* L.) is often infected with the clavicipitaceaes endophytic fungus *Acremonium lolii* (Latch, Christensen, and Samuels). The first discovery established a link between the present of *A. lolii* in perennial ryegrass and ryegrass staggers, a neurological disorder, sometimes seen in livestock grazing on ryegrass-dominant pastures. A number of alkaloids, such as loliterm B, are produced by *A. lolii* in association with the ryegrass. It was found that ryegrass staggers was caused largely by the presence of loliterm B. The combination between the endophyte associated within the host grass and production of these alkaloids gave rise to toxic activity to livestock disorders (12, 53,54).

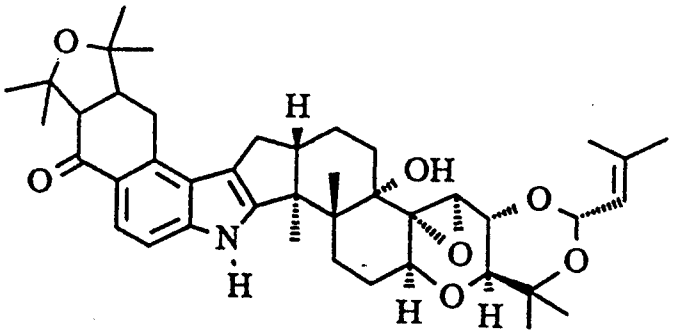
Ball *et al.* (53) found metabolites produced from *A. lolii* as two of its alkaloid metabolites, loliterm B and peramine (Figure 5) were produced and stored in perennial ryegrass (54). *A. lolii,* loliterm B, and peramine concentrations were found to be lowest in winter but highest in early summer when the plant was developing its reproductive structures. Loliterm B concentrations were highest in the basal part of the plant whereas peramine concentrations were highest in the upper part of the plant.

Mites and colleagues (55) discovered that the endophyte *Echinopogon* spp. caused a major problem in livestock in Australia. This endophyte caused symptoms similar to those of perennial ryegrass staggers. They observed that the fungus was located in the intercellular spaces of the leaves and seed of New Zealand and Australian specimens of *Echinopogon ovatus* (Figure 6). This fungus is serologically related to *Neotyphodium lolii* (the endophyte of perennial ryegrass) and other *Epichloe* and *Neotyphodium* species. The *in vitro* studies showed that these endophytes produced peramine and indole-diterpenoid

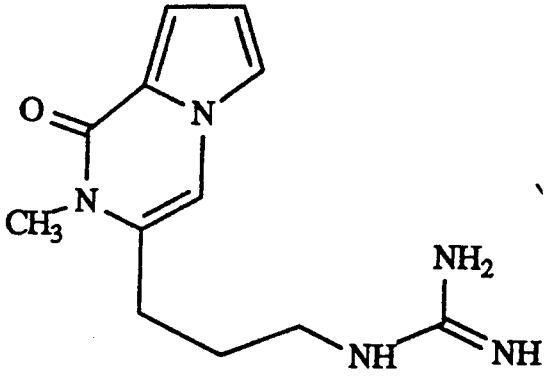
in the infected specimens of grass (55). They used ELISA and gas chromatography for detecting those compounds. They could not be detected in endophyte-free specimens.

Krohn and colleagues (56) investigated the constituents of an endophytic fungus from the *Mycelia sterilia* isolated following surface sterlization from the root of *Atropa belladonna.* Since the fungus did not sporulate it could not be characterized taxonomically. The culture extract of the fungus was found to have antibacterial and antifungal properties. The fungus was cultivated for 70 days at ambient temperature on two different media, biomalt and malt-soya semisolid agar. The combined cultures were homogenized and extracted. Then a crude compound was purified, crystallized, structurally elucidated, and analyzed for absolute configuration. Finally, the compound preussomerin was described (Figure 7), this compound showed activities against bacteria (*Bacillus megaterium*) and fungi. It also showed activity against many plant pathogenic organisms, as complied in Table 3 (56).

Li *et al.* (57) isolated an endophytic fungus *Cryptosporipsis cf. quercina* from the inner bark of *Triptergyium wilfordii.* The fungus produced a metabolite cryptocin (Figure 8) in oat seed culture broth. Cryptocin possesses impressive biological activity against certain plant pathogenic fungi such as *Pythium ultimum*, *Phytophthora spp.*, and *Sclerotinia*, as shown in Table 4. It was also active against some of human pathogens including *Candida albicans*, *C. parapsilosis*, *Histoplasma capsulatum* and *Cryptococcus neoformans* (57). Thereby the biological rational would suggest that *C. quercina* may be acting as a symbiotic endophyte that provides protection to its plant host.



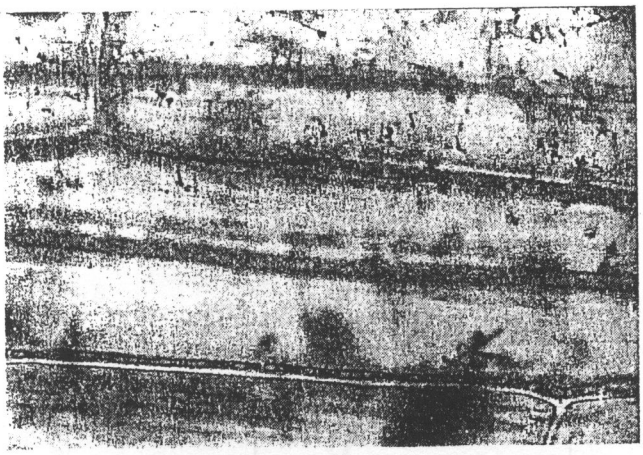
LOLITREM B



PERAMINE

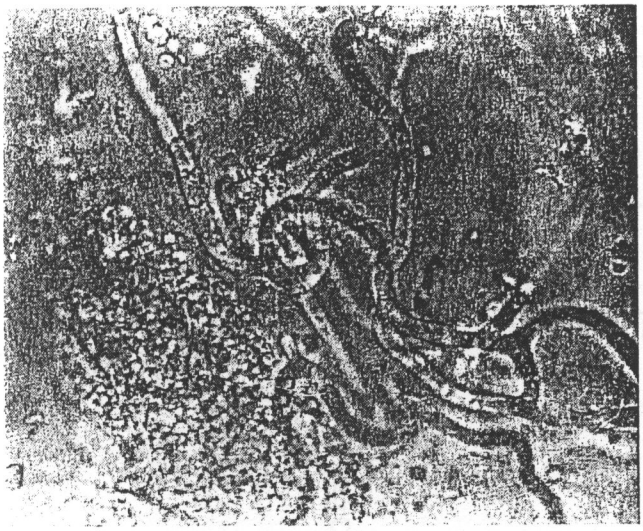
Figure 5. Alkaloid metabolites, loliterm B and peramine (54).

A



Photomicrograph of endophyte mycelium in the leaf sheath of *E. ovatus*.

B



Photomicrograph of endophyte mycelium in the aleurone layer of a seed of *E. ovatus*.

Figure 6. The endophyte of perennial ryegrass, observed in the intercellular spaces of leaves (**A**) and seeds (**B**) of New Zealand and Australian specimens of *Echinopogon ovatus* (55).

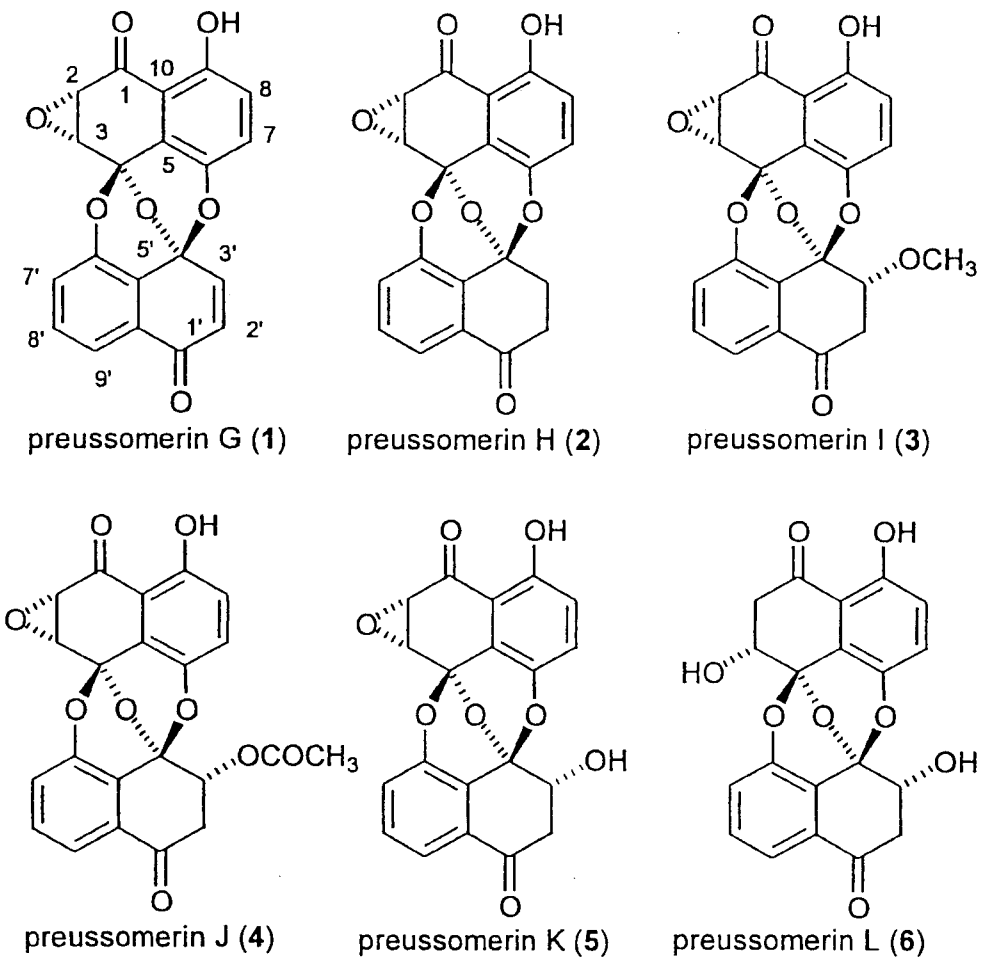


Figure 7. Preussomerin and its derivatives from a fungus endophyte in the *Mycelia sterilia* (56).

Table 3. Biological activities of preussomerins 1-6 (structure in Figure 7) against microbial test organisms by agar diffusion assay (56).

Table 3. Biological activity of preussomerins **1–6** against microbial test organisms, agar diffusion assay

Compound	Bm.	Chl.	Eur.	Fu.	Mm.	Mv.
1	0.6	0	0.9	1.1	0.1	0.6–1
2	0.3–0.5	0.1–0.2	–	–	–	0.8–1.3
3	0.2	0.3	0.5–0.6	–	0.1	0.4–0.8
4	0.4–0.5	0.1	0.8	–	–	0.8–1.2
5	0.5–0.7	0.5	0.5	–	–	1.1
6	0.2–0.5	0	–	0	0.2	0.4

Concentration: 50 µl of the given 2 mg/ml solution per plate; test organisms: Bm.=*Bacillus megaterium*, Mv.=*Microbotryum violaceum*, Mm.=*Mycotypha microspora*, Eur.=*Eurotium repens*, Fu.=*Fusarium oxysporum*, Chl.=*Chlorella fusca*.

Figure 8. The structure formula of cryptocin (57)

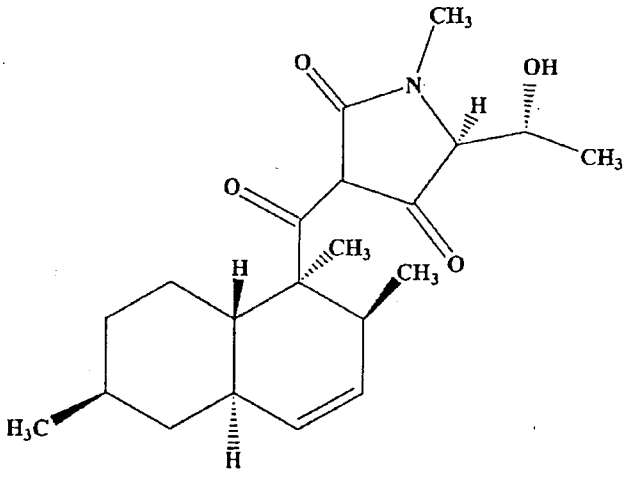


Table 4. Minimum inhibitory concentrations of cryptocin for representative groups of plant pathogenic fungi (57)

Minimum Inhibitory Concentrations of Cryptocin and Pseudomycin A for a Representative Group of Plant Pathogenic Fungi. The Family Name of Each Organism Tested Is Presented Next to the Specific Fungal Name in the Table

fungus (genus and species)	fungal family	cryptocin, μg/mL	pseudomycin A, μg/mL
Pythium ultimum	oomycete	0.78	>12.0
Phytophthora cinnamoni	oomycete	0.78	not active
Phytophthora citrophthora	oomycete	1.56	not active
Sclerotinia sclerotiorum	ascomycete	0.78	1.5
Pyricularia oryzae	ascomycete	0.39	>12.0
Rhizoctonia solani	basidiomycete	6.25	1.5
Geotrichum candidum	fungi imperfecti	1.56	1.5
Fusarium oxysporum	fungi imperfecti	1.56	>12.0

Toxins from fungal endophytes also have the potential of being insecticides. Cytotoxicity and insecticidal activity of endophytic fungi has been observed by Johnson and Whitney (58). Endophytic isolates were obtained from black spruce (*Picea mariana*) needles, then grown in liquid culture and culture broth extracted whereas fungal mass was frozen dried, and ground to a powder. Bioassay of the extracts was tested on spruce budworm (*Choristoneura fumiferana* Clem) cells cultures. While mycelium powder was fed to test budworm larvae. The data showed that crude extracts of some strains of *Cryptocline abietina*, *Aureobasidium pullulans* and *Hormonema dematioides* were active against to insect cells, whereas hyphae from *C. abietina* showed a reduction in dry mass and a retardation development when fed to larval (58).

In Australia, infection with the fungus *Phomopsis leptostromiformis* of lupin stubbles in pods and seeds was a major factor limitting utilisation due to the relatively high incidence of lupinosis in sheep. Lupinosis is a disorder in the liver of sheep grazing lupin stubble resulting in ill-thrift, photosensitization, jaundice and death. The causative agent, phomopsin A, interferes with microtubule-dependent intracellular transport mechanisms and acts as anti-mitotic agent in the liver (42). Hepatic damage results in hyperammonaemia, which in turn may induce degenerative changes in the central nervous system. Affected sheep may appear disorientated. The mycotoxin, phomopsin A is considered to be the primary toxic agent. Its pathogenic mechanism is however under investigation (42).

Endophytes can act as a plant-growth promoting agents. However, there is very little knowledge concerning plant-growth promoting cultivable root endophytes. Most studies have been conducted on mycorrhizal fungi. Abuscular mycorrhizal (AM) fungi occur on the roots of 80% of vascular flowering plant species. They are obligate biotrophs and cannot be cultured without the plant host. In contrast, a root endophyte can be easily grown on various complex and minimal substrates. Varma *et al.* (59) determined the pattern of root-culture colonization by *Piriformospora indica* (a root fungal endophyte) and assessed its effect on the growth of six plant species. The results showed that after roots of all six plants were colonized with *P. indica*, growth and biomass production could be promoted. This fungus provides a model for the study of beneficial plant-microbe interactions and a new tool for improving plant production systems. Furthermore, selected strains of *Epicoccum purpuraseens* and *Aureobasidum pullulans* could be related to the production of auxin-like factors because production of indole-3-acetic acid (IAA) and indole-3-acetonitrile has been demonstrated *in vitro.* Another plant hormone or cytokinins (kinetin) has been produced by the endophytic fungus *Hypoxylon* sp. isolated from tobacco. It stimulated the flowering process of its host (59).

6. Identification of Fungi.

The correct identification of fungi is of great practical importance not only in a clinical setting but also in plant associations, biodeterioration, biotechnology, and environmental studies. An enormous number of species of fungi are already known, and so taxonomists are being kept very busy with recognizing and describing new species and

grouping taxa. The limitation of classification in many fungi is that it is based mainly on morphology .

Some groups of fungi have economical or pathological importance and so have been studied more extensively. With these, other features beside morphology have been used in classification and identification. They include susceptibilities to yeast killer toxins, susceptibilities to chemical and antifungal drugs, molecular characters, physiological and biochemical characters, secondary metabolites produced, fatty acid composition, to name a few, have been used. The increased use and availability of molecular techniques have opened up many new areas within systematics and have enabled more traditional methods to be developed further.

6.1 Morphological identification

Classification systems of organisms are historically based on observed characteristics. This is the phenotypic approach that requires the use of a light microscope. The growth of isolates in appropriate culture media, enabling their most characteristic features to be recognized, is still the most common procedure used in practice. After a few weeks incubation, assignment of morphological species can be based on colony surface textures, hyphal pigments, exudates, margin shapes, growth rates, and sporulating structures. With this simplistic and mechanical approach, comparisons in floristic composition and species frequency can still be made among small groups of related samples without needing to name the isolate. But applications of this approach are limited because the kinds of taxa involved can not be compared over time or

communicated with other researchers. Because fungal names are tied to sporulating structures and because the majority of plant inhabiting fungi have been named based on structures produced on the host, examination of the host for fungi is a logical starting point. Collection of recently dead twigs, stems and leaves and litter should be examined for sporulation structures. Senescent or dead plant parts sometimes can be rehydrated and incubated in moist chambers to resume active sporulations. Moist chamber incubation or slow drying of living host tissues to induce development of lesions and emergence of sporulating structures can be carried out in parallel with isolations on nutrient agar to induce sporulation on host tissue (60).

Incubation of woody stems in plastic bags impedes desiccation and can permit mycelial out growth and identification or isolation of mycelium from colonized regions of decorticated stems or external fruiting on corticated stem (61). The cut ends of limbs and branches can be sealed to impede desiccation and then incubated to permit development of fruiting structures on the wood surface.

Anderson and French (62) observed the development of *Hypoxylon mammatum* on field-collected stems of *Populus tremulodes*. *Hypoxylon* cankers developed on stem sections that were coated with paraffin and incubated in sterile wet sand in greenhouses or incubators.

Chapela (63) and Boddy (64) used a similar approach to determine the effect of desiccation on colonization patterns by latent fungi in cut limbs. Distilled water agar slabs sealed in moistened cotton wool and plastic film or cotton wool and punctured

plastic film alone were applied to cut stem ends to impede drying during incubation in environmental cabinets or greenhouses.

Washed leaf discs of *Populus tremuloides* were incubated in moist chambers. The fungi developing on leaf discs were compared with those obtained from surface-sterile discs planted on malt agar (28). Chemical induction of senescence with paraquat may also provoke sporulation on the host (65). Girdling (ring-barking) a living stem may release latent endophytes in bark and sapwood and encourage their development *in situ.*

Sudden death of oaks caused by oak wilt or girdling, provided the opportunity to characterize the life cycle of *Hypoxylon punctulatum* (Berk& Ravenel) Cooke, a primary colonizer of recently killed oak stems. Barnett (66) observed the intimate association of the *Basidiobotrys* (now classified in *Xylocladium*) state with emerging stromata and proved their connection through cultural studies. The conidial state developed on internal pillars within the stromata and could be isolated from wood beneath the bark. Emergent stromata developed from deep (up to 6 mm.) in the bark. After following the ontogeny of the stromata, a mechanism was proposed of how gelatinous tissues inside the stromata expanded to eject the outer bark so as to expose the fertile perithecial layer.

During prolonged incubation of twigs and leaf sections on primary isolation plates, some taxa will preferentially sporulate on host tissue (Figure 3, 4, 9). Extended incubation of twig and needle sections permitted development of apothecia of *Chloroscypha* spp. in culture (67). Unknown sporulating structures observed in cultures, either on plated host tissue or produced on agar can be matched with those found on host

tissues in nature. *Rhabdocline parkeri* was first observed as sporodochia protruding through stromata and apothecia forming on surface-sterilized needles of *Pseudotsuga menziesii* in culture. Later, sporodochia were compared with structures collected on galled needles infested by the gall midge, *Cortarinia pseudotsujae* (Condrashoff), and with apothecia forming naturally on recently abscised needles (68).

Sridhar and Barlocher (69) observed formation of the teleomorph of *Heliscus lugdunensis* Sacc & Therry after 40 days of incubation after being subcultured from bark of *Picca glauca* (Moech.) Voss. Inoculating bark cultures on host tissue and other plant material may induce sporulation, allow for more natural development of sporulation, or permit observation of teleomorphs or synanamorphs not observed on agar (18, 69, 70).

Morphological studies of endophytic mycelia in seeds of five *Lolium* species (grass) revealed that considerable variation could occur in endophyte morphology in different host species (71). Despite the variation in morphological characteristics, there was still considerable overlap in endophyte morphologies across host species. Controlled experiments showed that the environmental conditions under which host plants are grown has considerable influence on hyphal diameters and the rates that endophytic hyphae of perennial ryegrass (*L. perenne* L.) enter leaf sheaths from seeds.

Light intensity, soil fertility, and volume of growth media appeared to particularly influence endophyte growth and colonization of its host.

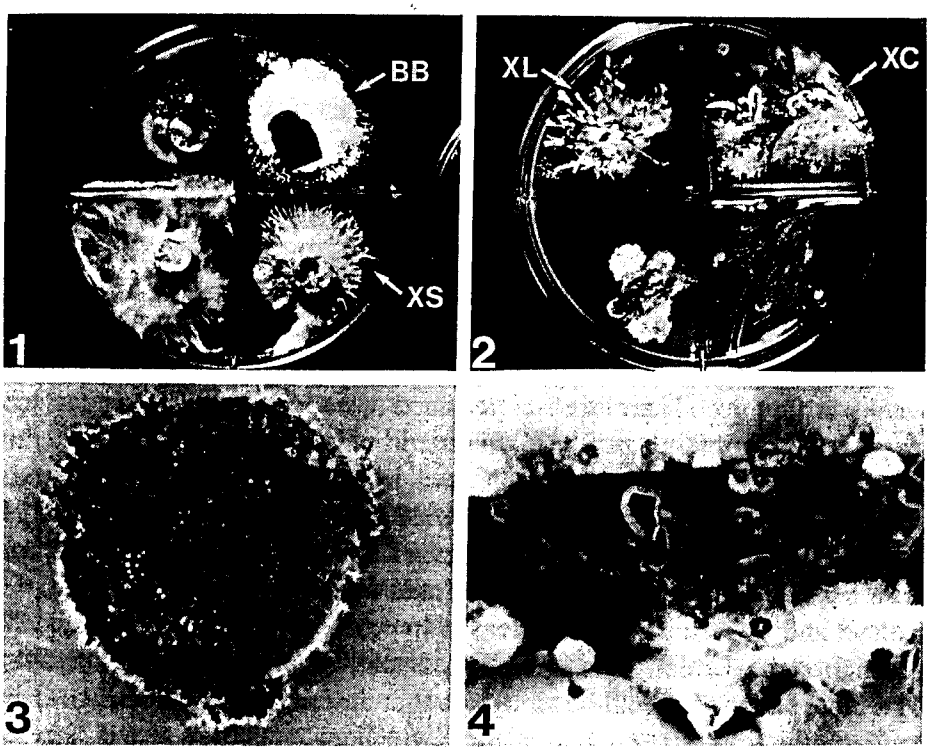


Figure 9. Sporulation of endophytic fungi on different types of healthy plant tissue in agar culture. **1.** Endophytes emerging from bark discs of *Carpinus caroliniana* after two weeks on malt yeast extract agar. BB = *Beauveria bassiana*, XS = unidentified *Xylaria* anamorph. **2.** Endophytes emerging from leaf sections of *Chamaecyparis thyoides* after three weeks on Mycosel agar. XL = anamorph of *Xylaria longipes* Nitschke, XC = anamorph of *Xylaria cubensis* (Mont.) Fr. **3.** Leaf disc (5 mm) of *Ilex opaca* with emergent conidiomata of *Phyllosticta concentrica* after two weeks on malt yeast agar with 4 mg/L cyclosporin A. **4.** Conidiomata of *Cytospora salicis* (Corda) Rabenh. developing from twig section of *Salix alba* L (23).

The amount of mycelium in many plant inventory accessions often was much lower than amounts observed in commercial cultivars. The low level of mycelium and infection rates in some accessions made detection difficult without relatively large sample sizes. In general, conditions favoring growth and vigor of the host also tended to support more vigorous growth of the endophyte. Furthermore, the systemic distribution of endophytic hyphae in host tissues depended on the particular host-endophyte association. For example, the endophytes of annual ryegrass species, unlike perennial ryegrass, poorly colonized leaf sheaths, but hyphae were found primarily in clump piths, especially in the inter-nodes. Similar results were reported by Latch *et al.* (72).

Many species of endophytes will not sporulate in agar culture, while with cultures of many others, the ability to sporulate deteriorates rapidly with successive transfers. In other cases, the time needed for spouration may be weeks or months. Therefore isolates need to be identified and characterized as soon as possible. Carroll (73) asserted that a century's "accumulated lore on manipulation of growth media and laboratory environment to induce sporulation" in endophytic isolates should be applied to sterile isolates. This approach may be valuable if one knows beforehand something of the isolate, and if there is sufficient reason to proceed with the tedious screening of each single isolate though countless media and incubation conditions. The ideal situation would be similar to that described by Barnett (74) where a semi-synthetic glucose-phenylalanine medium was developed that was suitable not only for direct isolation of the oak-wilt fungus from living wood but also promoted its rapid sporulation and identification. Even when there is potential economic incentive to experiment with growth conditions, such as

an isolate producing an interesting antibiotic, many sterile isolates are doomed to remain sterile.

6.2 Molecular identification.

Since the distinguishing morphological characteristics of a fungus are frequently too limited to allow its identification, physiological and biochemical techniques are applied. However, for the poorly differentiated filamentous fungi, these methods are laborious, time consuming, and somewhat variable and provide insufficient taxonomic resolution. In contrast, molecular methods are universally applicable. Comprehensive and detailed reviews of the use of molecular techniques in fungus systematics have been provided.

Two important technical advances have stimulated the use of molecular techniques. Firstly, the advent of PCR has allowed the analysis of small numbers of fungal cells or even single spores, dried herbarium material (40), or extinct organisms (75). Second, the selection of universal oligonucleotide primers specific to fungi (76, 77, 78, 79) has provided easy access to nucleotide sequences.

The aim of molecular studies in biodiversity is fourfold:

(i) phylogenetic studies, *i.e.*, tracing back the most probable course of evolution and the historic coherence between groups at higher taxonomic ranks;

(ii) taxomomic studies, mostly at the level of genera and species;

(iii) diagnostic applications, *i.e.*, recognition of defined taxonomic entities; and

(iv) epidemiology and population genetics, *i.e.*, monitoring outbreaks of subspecific entities with respect to the analysis of broad aims and levels of diversity has its own set of optimal techniques.

In this review, only phylogenic and taxonomic studies are discussed. The main reasons for the popularity of ribosomal DNA (rDNA) are that it is a multiple-copy, non-protein coding gene, whose repeated copies in tandem are homogenized by concerted evolution, and it is therefore almost always treated as a single- locus gene. Furthermore, ribosomes are present in all organisms, with a common evolutionary origin. Parts of the molecule are highly conserved (80, 81) and serve as reference points for evolutionary divergence studies.

6.2.1 Taxonomy and characterization of fungi by PCR.

Random PCR approaches are being increasingly used to generate molecular markers which are useful for taxonomy and for characterizing fungal populations. The main advantage of these approaches is that previous knowledge of DNA sequences is not required, so that any random primer can be tested to amplify any fungal DNA. RAPD primers are chosen empirically and tested to find RAPD banding patterns that are polymorphic between the taxa studied. The RAPD method has been successfully used to differentiate and identify fungi at the intraspecific level (82, 83, 84) and the interspecific level (85). Similarly, PCR fingerprinting with primers that detect hypervariable and

repeated sequences has been used to clarify the taxonomic relationships among both fungal strains and species (86). As both RAPD and inter-repeat PCR amplify DNA from non-specific primers, they need a pure DNA template and cannot be used to detect fungi in mixed samples.

More recently, AFLP fingerprinting has been developed to evaluate polymorphisms among various organisms, and this has already been applied to the detection of inter- and intraspecific genetic variation in fungi (87, 88). AFLP has several advantages over RAPD in terms of reproducibility and the level of resolution per reaction, the method has great potential for revealing variations among many fungi, especially at the intraspecific level.

Moon *et al.* (89) identified Epichloe endophytes (a group of filamentous fungi including both sexual *Epichloe* and asexual *Neotyphodium* species) in plants by a microsatellite-based PCR fingerprinting assay with automated analysis. This result showed a unique microsatellite loci of DIG-labeled dinucleotide and trinucleotide repeat probes. The probe was use to design primers for each locus of endophytes from different taxonomic groups. The assay was used successfully to identify a set of endophytes in plants and a reference database of allele sizes has been established for the panel of endophytes examined. This will expand, as new strains are analyzed (89).

6.2.2 Ribosomal DNA gene studies

The DNA sequences that encode ribosomal RNAs have been extensively used to study the taxonomic relationships and genetic variations in fungi (90, 91). The ribosomal RNA gene cluster is found in both nuclei and mitochondria, and consists of highly conserved and variable regions, which include the genes for the small 5.8S, and large rRNA subunits (79). The fungal nuclear rRNA genes are arranged as tandem repeats with several hundred copies per genome. The conserved sequences found in the large subunit (LSU) and small subunit (SSU) genes have been exploited to study the many relationships among distantly related fungi (90, 92, 93). The spacer regions between the subunits, called the internal transcribed spacers (ITS), and between the gene clusters, called the intergenic spacers (IGS), are considerably more variable than the subunit sequences, and have been used widely in studies on the relationships among species within a single genus or among intraspecific populations (94, 95, 96, 97, 98).

The amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics had been studied by White et al. (79). Locations of the specific-primers of the gene cluster on nuclear and mitochondrial rDNAs are shown in Figure 10 (79). The recommended primers for amplification of fungal rRNA genes are shown in Table 5.

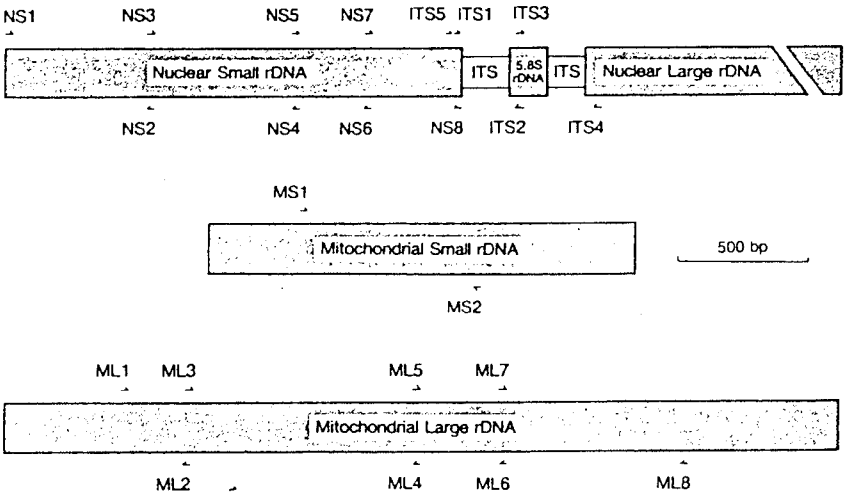


Figure 1 Locations on nuclear and mitochondrial rDNAs of PCR primers given in Table 1. The arrowheads represent the 3' end of each primer. The nuclear large rDNA is truncated in this figure.

Figure 10 Location on nuclear and mitochondrial rDNAs of primers given in Table 5. The arrowheads represent the 3' end of each primer. The nuclear large rDNA is truncated in this diagram (79).

Table 5. Primers for amplification of fungal ribosomal RNA genes (79).

Primers for Amplification of Fungal Ribosomal RNA Genes

rRNA	GenePrimer[a]	Product Size (bp)[b]	T_m (°C)[c]
Nuclear, small			
NS1	GTAGTCATATGCTTGTCTC	555	56
NS2	GGCTGCTGGCACCAGACTTGC		68
NS3	GCAAGTCTGGTGCCAGCAGCC	597	68
NS4	CTTCCGTCAATTCCTTTAAG		56
NS5	AACTTAAAGGAATTGACGGAAG	310	57
NS6	GCATCACAGACCTGTTATTGCCTC		65
NS7	GAGGCAATAACAGGTCTGTGATGC	377	65
NS8	TCCGCAGGTTCACCTACGGA		65
Nuclear, ITS			
ITS1	TCCGTAGGTGAACCTGCGG	290	65
ITS5	GGAAGTAAAAGTCGTAACAAGG	315	63
ITS2	GCTGCGTTCTTCATCGATGC	290	62
ITS3	GCATCGATGAAGAACGCAGC	330	62
ITS4	TCCTCCGCTTATTGATATGC		58
Mitochondrial, small			
MS1	CAGCAGTCAAGAATATTAGTCAATG	716	65
MS2	GCGGATTATCGAATTAAATAAC		63
Mitochondrial, large			
ML1	GTACTTTTGCATAATGGGTCAGC	253	68
ML2	TATGTTTCGTAGAAAACCAGC		63
ML3	GCTGGTTTTCTACGAAACATATTTAAG	934	67
ML4	GAGGATAATTTGCCGAGTTCC		68
ML5	CTCGGCAAATTATCCTCATAAG	359	66
ML6	CAGTAGAAGCTGCATAGGGTC		65
ML7	GACCCTATGCAGCTTCTACTG	735	63
ML8	TTATCCCTAGCGTAACTTTTATC		57

[a] All odd-numbered primers are 5′ primers; even numbers indicate 3′ primers. Sequences are written 5′-3′.

[b] Product sizes are approximate based on the rRNA genes of *S. cerevisiae*; the size of the region amplified is the product size minus the primers. Primers NS3 and NS4 amplify mitochondrial and bacterial rDNA from some organisms; expected product sizes are approximately 365 bp and 425 bp, respectively (see Fig. 2, lane 5).

[c] T_m's were calculated by the method of Meinkoth and Wahl, 1984.

6.2.2.1 Spacer regions

There are now many examples of the use of either RFLP or sequence differences in the different spacer regions for discriminating between closely related species within fungal genera. Most fungal phylogenetic studies have used sequences from the ribosome gene cluster. The structural regions coding for the 5.8S, 18S, and 28S ribosomal RNA genes were transcribed into RNA molecules that from part of the ribosome. Interspersed between the genes, spacer regions can also be transcribed. The ones that lie between the 18S and 5.8S, and the 5.8S and 28S genes are called the internally transcribed spacers, or ITS, while the region that separates the clusters of three genes along the chromosome is called the non-transcribed spacer or NTS. Just prior to where the 18S gene is transcribed there is another small spacer region called the externally transcribed spacer or ETS. Together, the ETS and NTS regions comprise the intergenic spacer region or IGS. The tree regions, structural genes, transcribed spacers and non- transcribed spacers, evolve at different rates, and their sequences can be used to discriminate between taxa at different levels. The ITS and IGS regions are much more variable and can be used to separate taxa from classes to species. On the other hand, the structural genes are so conserved that sequences present in these genes can be found in many organisms and can be used to delineate species to their order (99, 100).

6.2.2.2 ITS region

The ITS consists of two non-coding variable regions that are located within the rDNA repeats between the highly conserved small subunit, the 5.8S subunit, and the large subunit rRNA genes. The ITS region is a particularly useful area for molecular characterization studies in fungi for four main reasons:

(i) the ITS region is relatively short (500-800 bp) and can be easily amplified by PCR using universal single primer pairs that are complementary to conserved regions within the rRNA subunit genes (78);

(ii) the multicopy nature of the rDNA repeat makes the ITS region easy to amplify from small, dilute or highly degraded DNA samples (101);

(iii) the ITS region may be highly variable among morphologically distinct species (101, 102, 103, 104) and so ITS-generated RFLP restriction data can be used to estimate genetic distances and provide characters for systematic and phylogenetic analysis (105); and

(iv) PCR-generated ITS species-specific probes can be produced quickly, without the need to produce a chromosomal library (106) and many researchers have selected sequences from the ITS region to develop species-specific probes because the sequences occur in multiple copies and tend to be similar within and variable between fungal species.

Hamelin *et al.* (107) identified the root rot fungi by nested multiplex PCR technique. They used the sequences of ITS include 5.8S of rDNA subunit of *Cylindrocladium floridanum* and *Cylindrocarpon destructans*. Sequences were aligned

and compared with sequences of other fungi in GenBank. The species-specific primers amplified PCR products showed a variable sequence of ITS region and including the conserved sequence of 5.8S coding region. A specific sequence was found to have a potential for the development to an efficient and reliable detection method based on nested PCR for identification and detection of these fungi.

Jackson *et al.* (108) used the RFLP technique to analyse the ITS in rDNA of the dermatophytic fungus *Trichophyton rubrum.* The polymorphisms of this region detected the *T. rubrum* genomic DNAs by an amplified probe from the small-subunit (18S) rDNA. The result was defining both for species identification and strain differentiation of dermatophyte grouping.

6.2.2.3 IGS region

In contrast to other regions, fewer studies have considered the IGS region. Arora *et al.* (109) used RFLP derived from the IGS, located between the rRNA gene cluster, to determine variability within the species *Verticillium chlamydosporium* and other closely related species. They found that there was, in general, a low level of heterogeneity in this region within species, and that distinct IGS types could be associated with particular species. The IGS region has also been investigated in *Fusarium* and Appel and Gordon (110) found heterogeneity in this region in *F. oxysporum* on the basis of RFLPs of the PCR amplified product. Further studies on the IGS region have been undertaken with *Pythium ultimum*, where length heterogeneity was found to be due to subrepeat arrays (111).

6.3 Other identification techniques

6.3.1 Physiological and biochemical techniques

Because numerous fungi grow relatively rapidly in pure culture, it is possible to use physiological and biochemical techniques to identify and classify them (41, 112, 113). These techniques have been successfully used in study of black yeasts (114, 115, 116). The different ranges of growth temperature have been used as a complementary tool in the identification of both asexual (117) and sexual (118) fungi. Growth rates on defined media under controlled conditions are also valuable in studies of complex genera such as *Penicillium* (119). Commercially available kits such as the API system have also been used to identify filamentous fungi (120, 121). Paterson and Bridge (41) have published a compilation of the physiological techniques used in the identification of filamentous fungi. They list a variety of biochemical methods, which range from simple agar-based tests to more sophisticated chromatographic and electrophoretic methods (41).

6.3.2 Secondary metabolite profiles

Secondary metabolites are compounds neither essential for growth nor key intermediates of the organism's basic metabolism but presumably play some other roles in the life of fungi. They are usually found as a mixture of closely related molecules with peculiar and rare chemical structures. The most common are steroids, terpenes, alkaloids, cyclopeptides, and coumarins, some of which are mycotoxins. The advent of

thin-layer chromatography, especially the simple technique of directly spotting thin-layer chromatography plates with small samples of culture cut from petri dishes, has made it possible to qualitatively assess secondary metabolites much faster than by conventional extraction, purification, and concentration techniques. This improvement has resulted in huge amounts of new secondary metabolite data, which is now being incorporated in databases (113, 122). The use of this method in fungi taxonomy has been questioned because the production of these compounds can be affected by environmental conditions. The identification in ascomycete systematics is well illustrated by the chemotaxonomic studies performed in the Eurotiales (123) and Xylariales (124). In these orders, individual species can often be recognized on the basis of particular metabolite profiles. Integrated approaches involving morphology, physiology, and secondary metabolites have been used in several attempts to clarify the systematics of some fungi (112, 125).

6.3.3 Fatty acid composition

Cellular fatty acid composition is routinely determined in bacteria systematics. Both the type of fatty acid present and its relative concentration are useful characteristics for separating taxa. Until recently, these techniques were only rarely used in fungal taxonomy. Although fewer different fatty acids are produced by fungi than by bacteria (126), these analyses are increasingly used for differentiating fungi (127, 128, 129, 130). Pyrolysis gas chromatography, pyrolysis mass spectrometry, gas chromatography, and partition aqueous polymer two-phase systems are among the numerous methods used to determine these compounds (129). Recently, gas

chromatography, combined with methods of multivariate statistical analysis, has successfully been used to study the fatty acids of numerous and varied filamentous fungi, including oomycetes, zygomycetes, basidiomycetes, and even sterile mycelia (129, 130). These techniques have also proved to be useful at intraspecific level. However, several technical aspects of the procedure used must be highly controlled to minimize sources of variation, which can influence the results enormously. The culture conditions and temperature are among the most important factors to be standardized (130).

6.3.4 Protein composition

Isoenzyme patterns produced by electrophoretic techniques (zymograms) have determined generic relationships and differentiated species (131). Apart from electrophoresis, immunological techniques and protein sequencing also provide suitable resolution for interspecific characterization (132). Isozyme analysis is considered to be an economical and practical technique for screening large populations. What is more, the characteristics determined by this technique are generally accepted to be of independent genetic origin (132). Allozyme (allelic isozyme) data are commonly used in phylogenetic studies (132).

CHAPTER III

MATERIALS AND METHODS

1. Medicinal Plant Samples

Healthy leaves and limbs were collected from medicinal plant species in various forest areas in Chiangmai, Songkhla, and Ubon Ratchathani provinces, Thailand. The cut ends of plant samples were sealed with parafilm. The collected plant samples were kept in sealed plastic bags and stored in a refrigerator. The samples were further processed in the laboratory.

2. Culture media

Culture media used for cultivation of endophytic fungi were Corn meal agar (CMA) (Difco), Malt extract agar (MEA) (Merck), Potato dextrose agar (PDA) (Difco), Sabouraud 4 % dextrose agar (SDA) (Merck), malt extract powder (Merck), yeast extract powder (Difco), and agar base (agar-agar ultrapure granulated, Merck). Other mycological media were Tap water agar (TWA), Yeast extract sucrose medium (agar and broth) (YES), and Malt Czapek broth (MCz), the formula are shown in Appendix 1.

The medium for growing bacteria was Mueller-Hinton medium (Merck). The medium base for growing TB was a complete Middlebrook 7H9 broth (7H9GC-tween) (Appendix 1) which was composed of Middlebrook 7H9 broth (Difco), glycerol (Sigma), Casitone (Difco), oleic acid, albumin, dextrose, and catalase (OADC) enrichment (BBL) and Lowenstein-Jensen (LJ) medium (Appendix 1). Complete RPMI 1640 for malarial culture was RPMI 1640 medium with L-glutamine (GIBCO) and others (Appendix 1).

3. Chemicals

The following chemical were used: Sucrose (commercial, food grade and AR; Merck), boric acid (Merck, GR), sorbitol (Merck, AR), sodium nitrate ($NaNO_3$) (BHD, AR), sodium chloride (NaCl) (Merck, GR), sodium hydrogencarbonate ($NaHCO_3$) (Merck, GR), sodium acetate (NaOAc) (Sigma, AR), sodium hypochlorite (NaOCl) (Clorox®, 5.25 % w/w available chlorine), disodium hydrogenphosphate (Na_2HPO_4) anhydrous (Merck, GR), potassium dihydrogenphosphate (KH_2PO_4) anhydrous (Merck, GR), magnesium chloride ($MgCl_2$) (Merck, GR) magnesium sulphate heptahydrate ($MgSO_4$ $7H_2O$) (Merck, GR), potassium chloride (KCl) (Riedel-deHaen, AR), dipotassium hydrogenphosphate (K_2HPO_4) (Merck, GR), zinc sulphate heptahydrate ($ZnSO_4$ $7H_2O$) (Merck, GR), copper sulphate pentahydrate ($CuSO_4$ $5H_2O$) (Merck, GR), Ferrous sulphate heptahydrate ($FeSO_4$ 7 H_2O) (Merck, GR), absolute ethanol (Merck, AR), 95% ethanol (industrial grade), liquid paraffin (specific gravity of 0.83-0.89, medicinal grade), dimethyl sulfoxide (DMSO) (Merck, AR), dichloromethane (CH_2Cl_2) (Labscan, AR), chloroform (CH_2Cl_2) (Merck, AR), phenol

(C_6H_5OH) (Merck, AR), Tris-HCl (Sigma), EDTA (Sigma, AR), polyoxyethylene sorbitan monooleate (Tween 80) (Sigma, Laboratory grade), *p*-iodonitrotetrazolium violet (INT, $C_{19}H_{13}ClIN_5O_2$) (Sigma, Laboratory grade), isoniazid (INH) (Sigma, AR), kanamicin (KM) (Sigma, AR), rifampin (RMP) (Sigma, AR), Alamar blue (MABA) (Gibco), gentamycin sulphate (pharmaceutical organization, Thailand) (medicinal grade), methylene blue (Sigma), Azure B (Sigma), Eosin (Sigma), Giemsa dye (Merck), glycerol (Merck, GR) and bromophenol blue (Sigma)

Molecular biology grade reagent used were dideoxynucleotide triphosphate (dATP,dCTP,dGTP, and dUTP) (Boehringer-Mannheim), *Taq* DNA polymerase (prepared in our laboratory), *Pst*I (Boehringer-Mannheim), ribonuclease A (Boehringer-Mannheim), and LE agarose (Seakem®, FMC).

4. Microorganisms

Bacteria used in antibacterial activity assays were *Staphylococcus aureus* ATCC29213, *Enterococcus faecalis* ATCC29212, *Bacillus subtilis* ATCC6633, *Escherichia coli* ATCC25922 and *Pseudomonas aeruginosa* ATCC27853. *Candida albicans* ATCC10231 and *Trichophyton mentagrophytes* (a clinical isolate from Dermatological Institute, Bangkok Thailand) were used in antifungal activity assays. *Mycobacterium tuberculosis* H37Ra ATCC25177 (H37Ra) obtained from the American Type Culture Collection (ATCC, Rockvill, MD) was used in anti Mycobacterium assays. In antimalarial assays *Plasmodium falciparum* K1 isolate was used. The strain was originally cultured and developed as an *in vitro* continuous strain

by Professor Sodsri Thaithong (Department of Biology, Faculty of Science, Chulalongkorn University).

5. Isolation of Fungal Endophytes

Cleaned leaves and 5-cm fragments of cleaned limbs of each plant sample were surface-sterilized by the method modified from Sieber *et al.* (133, 134). The plant samples were soaked in 70 % (v/v) ethanol for 1 min, followed with Clorox® for 5 min before rinsing with sterile distilled water 2 times. The surface-sterilized plant samples were allowed to dry briefly on sterile paper towels in sterile petri dishes.

Small pieces (8x8 mm) of leaf were cut from the middle rib and lamina, and placed onto 90-mm petri dishes containing tap water agar. Small and thin pieces of limb were cut from the middle part (1-cm long) of limb fragments and placed onto tap water agar plates. All petri dishes were incubated at room temperature and examined every day under a stereomicroscope for fungal growth.

Fungal endophytes germinating from the plant tissues were transferred to half strength malt extract agar (1/2 MEA) by hyphal tip transfer (133, 134). They were incubated for 7-14 days at 25 °C and purity was determined by colony morphology. Fungus isolates with different morphology were collected for further study.

6. Characterization of Endophytic Fungi

By using the hyphal tip isolation technique, each isolate was transferred onto five different media, including corn meal agar (CMA), malt extract agar (MEA),

potato dextrose agar (PDA), Sabouraud's dextrose agar (SDA), and yeast extract sucrose agar (YES). After cultivation for 14 days at room temperature they were photographed.

7. Preservation of Endophytic Fungi

Fungal endophyte isolates were kept at 4 °C by storage as agar slants under liquid paraffin or as agar blocks in sterile water as described by Smith and Onions (135). They were kept in duplicate.

7.1 Storage under liquid paraffin

Fungal endophyte isolates were grown on corn meal malt extract agar slant (CMMA) at 25 °C for 10 days. The mature cultures were then covered up to 10-mm height with sterile liquid paraffin and sealed. The liquid paraffin was steriled and by autoclaving twice at 121 °C for 15 min.

7.2 Storage in water.

Five-mm cubes were cut from the growing edge of fungal colonies grown on agar medium. Altogether, 5-7 pieces of agar blocks were put in 4-dram glass vials containing 5-ml sterile distilled water and sealed.

8. Fungal Cultivation and Metabolite Extraction

8.1 Fungal cultivation

Each fungal endophyte isolate was grown on MEA for 1-2 weeks depending on the individual fungal growth rate. After that, agar blocks of 5x5 mm dimension were cut off by sterile sharp blade. Malt Czapek broth (MCz) and Yeast extract sucrose broth (YES) were used to cultivate endophytic fungi for secondary metabolite production. Six pieces of agar cube were inoculated into each 1-L flask containing 200 ml of culture medium. Then, they were still cultured at 25 °C for 3 weeks or until the mycelium grew over the surface area.

8.2 Fungal metabolite extraction

The culture broth was filtered through 4 layers of cotton gauze and exhaustively pressed. The filtrate was extracted with an equal volume of dichloromethane (CH_2Cl_2) 3 times. The solvent was then evaporated and removed using a rotary evaporator (Buchi) at 40 °C. The crude extract was dissolved with dichloromethane, transferred into a vial and air dried. The dried crude extract was dissolved in dimethyl sulfoxide (DMSO) for bioassays.

The fungal mycelium, was washed in running tap water, packed in a glass bottle and lyophilized until complete dryness. The dried mycelium was grounded and extracted twice with dichloromethane-methanol (1:1). The extracted solvent was filtered through filter paper (Whatman No. 4). The filtrate was transferred to a glass vial and air dried. The dried crude extract was dissolved in DMSO for bioassays.

9. Determination of Antimicrobial Activities

Antimicrobial activities of crude extracts against test microorganisms were determined by broth microdilution test that was modified from the method described by Wood and Washington, and Ingroff and Pfaller (136, 137).

9.1 Test microorganisms

The test microorganisms are listed in Table 6 (below). These microorganisms represent reference strains for susceptibility testing under recommendations of the National Committee for Clinical Laboratory Standards (NCCLS).

Table 6. Test microorganisms for standardization and microdilution assay.

Type of microorganisms	**Reference strains**
Gram positive, cocci bacterium	*S. aureus* ATCC29213
Gram negative, cocci bacterium	*E. faecalis* ATCC29212
Gram positive, rod bacterium	*B. subtilis* ATCC6633
Gram negative, rod bacterium	*E. coli* ATCC25922
Gram negative, rod bacterium	*P. aeruginosa* ATCC27853
Fungus, yeast form	*C. albicans* ATCC10231
Fungus, filamentous form	*T. mentagrophytes* (clinical isolate)

9.2 Standardization of inoculum

The stocked bacteria were subcultured on Mueller-Hinton agar (MHA) plates. Suspension cultures were prepared by inoculating 4-5 fresh single colonies in 5 ml Mueller-Hinton broth (MHB) grown in a rotary shaker for 2-3 h, depending on the growth rate, at 37 °C. Inocula were prepared to match the turbidity of a 0.5 McFarland standard. By using a spectrophotometer, the related value of 0.1 optical density (OD) at 625 nm was obtained.

C. albicans was grown on Sabouraud dextrose agar (SDA) plates at 25 °C for 24-48 h. Five colonies, each at least 1 mm in diameter, were then picked and suspended in 5 ml of sterile normal saline (0.85 % NaCl). The turbidity of cell suspension was measured at 625 nm and adjusted to 0.1 OD.

T. mentagrophytes was grown on SDA slants at room temperature for 5-7 days until good conidia production was obtained. The slant washed with 5 ml of 0.05 % Tween 80 in normal saline, then vortexed and allowed to stand upright for 5 min for mycelial fragments to settle. The upper phase conidial suspension was then taken and suspended in normal saline in order to obtain turbidity of 0.1 OD at 625 nm.

All adjusted inocula were then ten-fold diluted for use in colony counting. Finally, colony forming units per ml (CFU)/ml were calculated and assumed to be the standard cell concentration corresponding to the turbidity of a 0.5 McFarland standard.

9.3 Preparation of bacterial inoculum

Bacterial inoculum was prepared according to the standard method (136). Bacteria were grown on MHA for 24 h at 37 °C. Selected fresh single colonies (4-5) were inoculated into 5 ml of MHB and incubated on a rotary shaker for 2-3 h at 37 °C.

The turbidity of the bacterial suspension was adjusted with sterile normal saline solution to match the turbidity of a 0.5 McFarland standard (OD 0.1 at 625 nm). Then the suspension was diluted with MHB to contain $2x10^3$ CFU/ml.

9.4 Preparation of fungal inoculum

Fungal inoculum was prepared according to the method described by Espinel-Ingroff and Pfaller (137). *C. albicans* and *T. mentagrophytes* were grown on SDA slant at 25 °C for 24 h and 5 days, respectively. Yeast cell suspensions were prepared in sterile normal saline solution and turbidity was adjusted to match that of a 0.5 McFarland standard. The final inoculum suspension was diluted with SDB to contain $2x10^3$ CFU/ml. Conidia of filamentous fungi were harvested by flooding the slant culture with 5 ml of sterile 0.05 % Tween 80 in normal saline solution. The conidial solution was suspended and hyphal fragments were allowed to settle for 5 min. The upper homogeneous suspension of conidia was pipetted and diluted with sterile 0.05 % Tween 80 in normal saline to match a 0.5 McFarland turbidity standard (OD 0.1 at 625 nm). The final inoculum was diluted with SDB to obtain a conidial suspension containing $2x10^3$ CFU/ml.

9.5 Assay procedure

Solution of crude extracts in DMSO (with varied concentrations in DMSO, which depending on the solubility of the crude extract and ranging between 5000 µg/ml to 625 µg/ml) was diluted with MHB and SDB for assays of antibacterial and antifungal activities, respectively. A 50-µl volume of broth containing crude extract was dispensed into each well of sterile multi well microdilution plates (96-flat-bottom

wells). The final adjusted microbial suspension was inoculated into each well in a volume of 50-µl (final inoculum size was 10^3 CFU/ml). A sterility control well was included in each plate. Sterile extract-free medium that contained the corresponding amount of DMSO, instead of the extract containing broth, was dispensed in the growth control well. A 100-µl volume of extract-free medium was used as the sterility control. Bacterium plates were incubated at 37 °C for 24 h. Fungus plates were incubated at 30 °C for 24 h and 3 days for *C. albicans* and *T. mentagrophytes*, respectively.

9.6 Reading of microplate assays

Antibacterial and anti-*Candida* activities were determined by developing a simple colorimetric method using *p*-iodonitrotetrazolium (INT), an oxidation-reduction indicator that measures metabolic activity of growing microbes. A 10-µl volume of 2-mM INT solution was added into each well. The mixtures were incubated for 1 h. Growth in each well was indicated by a color change from colorless to violet. Crude extracts with antimicrobial activity inhibited bacterial growth thus preventing the development of a violet color.

Anti-*T. mentagrophytes* activity was assessed using an inverted microscope. Crude extracts with antifungal activity inhibited or retarded growth of this filamentous fungus.

10. The Microdilution Assay of Compounds Against *Mycobacterium tuberculosis*

10.1 Preparation of mycobacteria inoculum

M. tuberculosis was maintained on slants of Lowenstein-Jensen (LJ) medium. Culture suspension was prepared by growing an initial inoculum on LJ agar plates, followed by subculture in complete Middlebrook 7H9 broth (7H9GC-tween). The culture suspension was incubated in 500-ml nephelometer flasks in a rotary shaker at 150 rpm at 37 °C until the optical density at 550 nm reached 0.4-0.5. Bacteria were washed and suspended in 20 ml of phosphate buffer saline and passed through an 8-μm pore-sized filter to eliminate cell clumps. The filtrates were aliquoted, stored at -80 °C and used within 30 days.

10.2 Preparation of control drugs

Isoniazid (INH), kanamycin (KM) and rifampicin (RMP) were prepared as stock solutions of 1 % in distilled water except for RMP that was dissolved in methanol. Drug stock solutions were filter sterilized (0.22-μm pore size) and stored at –80 °C for not more than 30 days. The minimal inhibitory concentration (MIC) values were determined continuously for four days for each experiment. A common set of frozen drug stock solutions was used for all experiments, with a previously unthawed aliquot being used for each experiment.

10.3 Test metabolite preparations

Fungal metabolites were taken from stock solutions in DMSO dissolved and diluted with the 7H9GC without Tween 80. The diluted metabolite dilutions were serially twofold diluted in 7H9GC (without Tween 80).

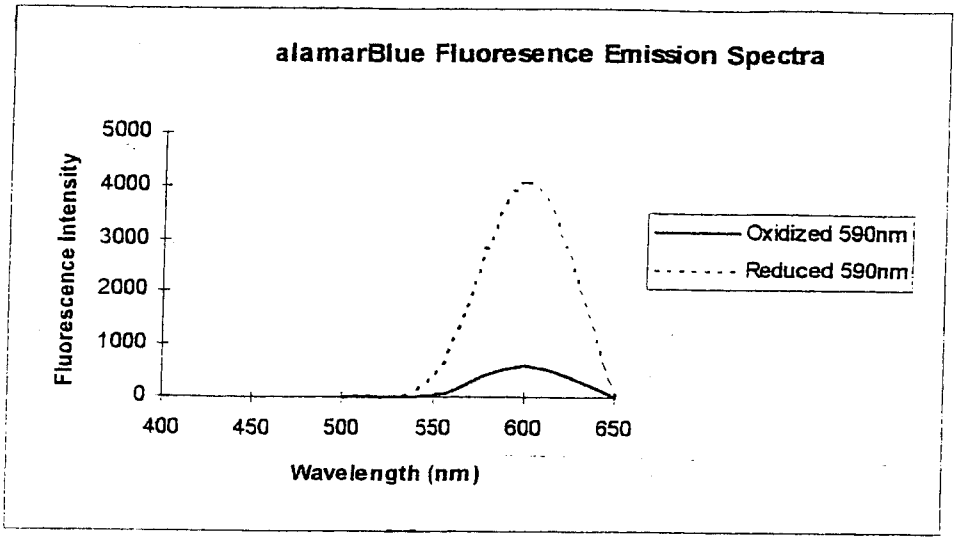
10.4 Alamar blue (MABA) assay for anti-TB activity

Anti-TB activity assay was performed in microplate 96-well (flat-bottom) plates according to the method described by Collins and Franzblau (138). Outer perimeter wells were filled with sterile water to prevent dehydration of interior experimental wells. Frozen inoculum was initially diluted to 1:20 in the 7H9GC (without Tween 80) medium followed by 1:50 dilution in 7H9GC (without Tween 80). The addition of 100 µl in a well resulted in the final bacterial titer of $5x10^4$ CFU/ml. Microplates were well sealed and incubated at 37 °C. On the fourth day of incubation, 20 µl of 10x Alamar blue solution and 12.5µl of 20% Tween 80 were added. The plates were reincubated at 37 °C. A color change from blue to red in wells could be observed at the 12^{th} and 24^{th} hours. Visual MICs were defined as the lowest concentration of crude extract that prevented a color change.

Alamar blue assay was designed to measure quantitatively the proliferation of bacteria. By incorporation of a fluorometric and colorimetric growth indicator, the assay was based on detection of metabolic activity. Continued growth maintained a reducing environment while inhibition of growth gave an oxidized environment. Reduction related to growth caused the redox indicator to change from oxidized (non-fluorescent, blue) to a reduced (fluorescent, red) form. Data could be collected using either fluorescent-based or adsorbance-based instrumentation.

Fluorescence was monitored at 560 nm excitation wavelength and 590 nm emission wavelength. Absorbance was monitored at 570 nm and 600 nm (Figure 11 A, B) (139).

A



B

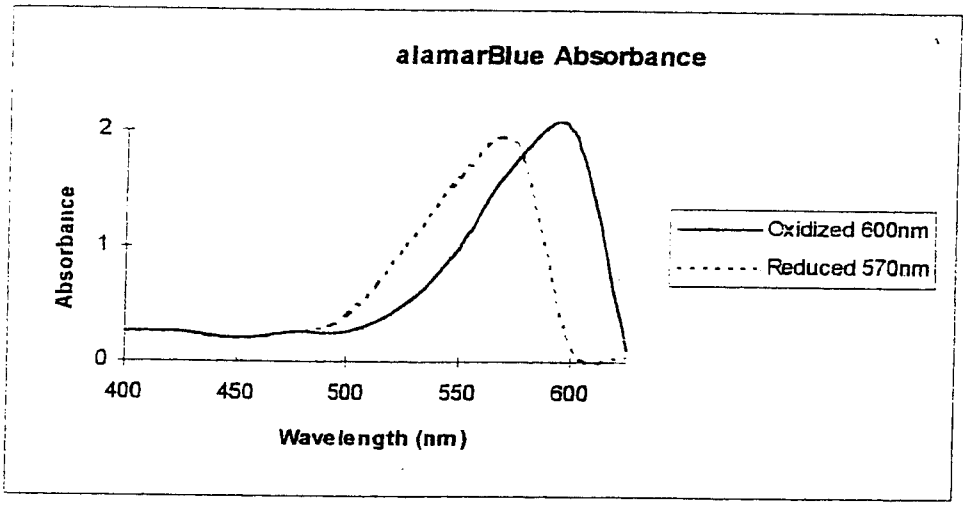


Figure 11. **A;**The Alamar-Blue fluoresence emission spectra. **B;** Alamar-Blue absorbance (139)

11. Antimalarial Activity Testing

11.1 Malaria parasites

P. falciparum K1 isolate was highly resistant to both chloroquine (MIC = $5x10^{-6}$ M) and pyrimethamine (MIC = $5x10^{-5}$ M). It was propagated by consecutive culturing as modified from the method originally described by Trager and Jensen (140).

11.2 Malaria cultivation supply

11.2.1 Culture medium

The medium was prepared by dissolving 10.40 g of RPMI 1640 medium and 5.94 g of HEPES buffer in 960 ml of distilled water, then adjusting to 1 liter. The medium was well mixed with a magnetic stirrer, and 2 ml of gentamicin sulfate (80 mg) was added lather before sterilization by filtration through a Millipore filter of 0.45 μm pore size. A 100-ml of sterilized medium was then transferred into a sterile glass bottle as stock medium, which could be stored up to 4 months at 4 °C (Appendix 1). To 100-ml medium stock 4.2 ml of 5 % (w/v) $NaHCO_3$ (Appendix 1) was added to adjust the medium to pH 7.4. This medium was referred to as complete medium (cRPMI). Before using media to cultivate parasites, 10-12 ml of human serum was added (10 % supplementation). This working solution was referred to as the complete RPMI medium with serum (cRPMI with serum). The solution prepared was stored at 4 °C and used within one week.

11.2.2 Normal human serum

Normal human serum of blood group types A, B and AB were collected from healthy donors who had no history of malarial infection, never been to the malarial endemic areas and never received any drug within 2 weeks before collection time. In general, the fresh blood was preserved in a sterile bag containing no anticoagulant and allowed to clot at room temperature for 30 min. The bag was then stored at 4 °C overnight to allow the complete clot. The clotted blood was centrifuged at 750 g for 30 min. After that the serum was transferred aseptically to a new sterile bag and inactivated at 56 °C for 30 min. Approximate 10-12 ml of fresh serum was distributed aseptically into sterile tubes, stored at –20 °C and was used within 3-6 months. Serum groups A and B were used for routine cultivation, whereas serum group AB was used to investigate blood schizontocidal activity *in vitro*.

11.2.3 Non-infected erythrocytes

Human erythrocytes were collected from healthy donors who possessed type "O" blood group, and who had no past experience of malarial infection. Approximately, 300 ml of blood was collected into a sterile bag containing citrate, phosphate and dextrose (CPD) solution as an anticoagulant. After thorough mixing, an appropriate volume of 40-50 ml of blood was dispensed aseptically into a sterile glass bottle and stored at 4 °C for using within one month. For cultivation, a 50 % non-infected red blood cells suspension was prepared as follows. Ten ml of whole red blood cells was transferred aseptically to a 15 ml centrifuge tube and centrifuged at 750 g for 15 min at 4 °C. The supernatant and puffy coat were removed. The packed red cells were washed twice with phosphate buffered saline (PBS, pH 7.4)

(Appendix 1). For use in cultivation, washed packed red cells were re-suspended with an equal volume of complete RPMI with serum to make up 50 % (v/v) of non-infected red blood cells.

11.3 *In vitro* cultivation of *P. falciparum* and maintenance of continuous culture

According to the continuous culturing technique described by Trager and Jensen (140), the parasites were cultured in RPMI 1640 medium with 10 % human serum in 60-mm diameter pertri dishes. Each dish contained 4 ml of 6 % red cell suspension, with an initial parasitaemia of 0.5-1.0 %. Petri dishes were placed in a candle jar (a glass desiccator equipped with stopcock and white candle). The white candle was lit and the cover was put on top with the stopcock open. When the lit candle was extinguished, the stopcock was immediately closed. By this method, the interior atmosphere with low oxygen *i.e.* 3 % O_2, 7 % CO_2 and 90 % N_2 was created. The candle jar was incubated in the incubator of 37-37.5 °C afterwards.

The medium was renewed daily. Old medium was removed with a pasteur pipette. The thin smear was made and stained with Field's stain (Appendix 1). Percentage of parasitaemia was estimated by counting the infected red cells in a total of 10,000 red cells. Subculturing was performed when parasitaemia was higher than 5-6 %. Briefly, infected red blood cells were harvested into a test tube and then centrifuged at 500 g for 7 min at 4 °C. The packed red cells were re-suspended to make 50 % cell suspension in cRPMI with serum. The parasitaemia was reduced to 1 % by diluting with 50 % non-infected red blood cells. Before dispensing the suspension into the petri dishes, cell suspension percentage was diluted from 60 % to

50 % cell suspensions by using cRPMI with serum. Approximate 4-5 ml of suspension was then placed into each dish and cultured as mentioned above.

11.4 Synchronization of the cultures

In general continuous cultures of *P. falciparum* led to loss of synchronicity. This could have complicated the evaluation of experimental results. Thus, it was important to begin experiments with a mixture of synchronous ring stage and late trophozoit at the ratio 1:1 following the method of Lambros and Vanderberg (141) to synchronize each culture line.

Briefly, a synchronous cultures of K1 with high parasitaemia, mostly ring stage, were pooled in a centrifuge tube and centrifuged at 500 g for 10 min at 4 °C. After the supernatant was removed, 5 volumes of 5 % (w/v) sterile sorbitol were added into the packed cells and gently mixing. The mixture was allowed to stand upright for 20 min at 37 °C. During this period of incubation, only erythrocytes harboring late trophozoites and schizonts were selectively lysed, leaving only the erythrocytes infected with ring stage among non-infected red blood cells. Finally, sorbitol solution was removed by centrifugation. The packed cells were washed twice with PBS and adjusted to 5-6 % of cell suspension and then cultured in the candle jar.

The synchronized ring stage culture was derived after 48 h of incubation whereas the synchronized late trophozoite culture required only 18-20 h for incubation. Synchronization of the culture lines was performed on different days at 24-hour intervals, in order to obtain different stages (rings and late trophozoites) on the same day. Synchronous rings and late trophozoites were mixed before using.

11.5 Cryopreservation

11.5.1 Freezing and storing

P. falciparum continuously cultured was harvested whenever > 5 % ring stage was observed. This culture was kept frozen as a stock culture. The culture suspension was centrifuged at 500 g for 7 min at 4 °C. The packed red cells were re-suspended with an equal volume of freezing solution (sorbitol, 0.9 % NaCl and 99 % glycerin) (Appendix 1) thereafter. Aliquots of 1-1.5 ml of the suspension were carefully transferred to cryopreservation tubes and frozen at –170 °C in a liquid nitrogen tank (142).

11.5.2 Thawing

Frozen cryopreserved tubes were thawed at 37 °C in a waterbath. The suspensions were transferred to a sterile centrifuge tubes, to which an equal volume of sterile 3.5 % NaCl solution was added by centrifugation at 350 g for 7 min at 4 °C. After gentle mixing by using a pasteur pipette, the suspension was centrifuged again. The supernatant was removed, followed by addition of an equal volume of human serum, gently mixing and centrifugation. The packed red cells were then washed twice with PBS under centrifugation. The final packed red cells were then re-suspended in cRPMI with serum to obtain 5 % suspension or less. The red cells were later dispensed into petri dished and used to culture in a candle jar. On the following day, fresh red cells were additionally supplied. Subculturing was performed whenever 4-5 % parasitaemia was detected.

11.6 Determination of antimalarial activity of fungal endophyte metabolites

11.6.1 Test metabolite preparation

Stock crude extracts in DMSO solutions were diluted with cRPMI to yield a concentration of 1000 µg/ml. Sterile extract-free medium containing the corresponding concentration of DMSO was used as control.

11.6.2 *In vitro* antimarial activity.

In vitro antimarial activity assay was performed in 96-well tissue culture plates (flat-bottom) which contained 100 µl erythrocyte suspension. Then 50-µl of each metabolite solution was put into each well of one row. Two-fold serial dilutions of the fungal metabolites were made with cRPMI 1640 medium using 50 µl dilutors. The 50 µl of parasitic erythrocytes (parasitemia of approximate 1 %) at 2 % cell suspension was added to each well. The microdilution plates were then incubated in a candle jar at 37 °C. To assess *in vitro* antimalarial activity using morphological comparison of parasites, cell pellets were removed from each well after 48-hour incubation. Thin smears of cell pellets were prepared and stained with Field's stain. Parasites of the tested well were morphologically compared with those of the control well (143,144,145, 146).

12. Endophytic Fungal DNA Extraction and PCR Amplification

12.1 Mycelium preparation

Selected fungal endophytes were grown on PDA plates. By using a sterile needle, young mycelium was transferred into 200-ml Erlenmeyer flask containing 25 ml PDB. Then the mycelium was incubated at 25 °C for 10-14 days. The growing mycelium was finally harvested by means of a pasteur pipette being bent at its tip to collect the mycelium. The mycelium was transferred to 50-ml centrifuge tubes, washed with sterile distilled water and centrifuged (Sorvall RC 5C plus) a 3000 g (5000 rpm, rotor SS-34) at 4 °C for 5 min. This process was repeated two more times. The supernatant was discarded. The pellet was then transferred into the suitable container for lyophilization. Dried pellet was manually ground into fine powder by using a mortar and pestle. The ground powder would be further subjected to DNA extraction.

12.2 DNA extraction

Fungal DNA extraction was performed according to the method described by Lee and Taylor (147). Ground mycelium (approximate 20-60 mg) was used to fill up to one third of a 1.5-ml microcentrifuge tube. Lysis buffer 400 µl (Appendix 1) was added to the tube, making the final volume up to 700 µl. The tube was then incubated at 65 °C for 1 hour with agitation. Then, 400 µl of chloroform: phenol (Appendix 1) was added to the mixture and the tube was inverted slowly. Microcentrifugation

(benchtop; Eppendrof) at 10,000 g was applied at room temperature for 15 min or until the top aqueous phase looked clear.

The aqueous phase was transferred to a fresh tube with the 3 M dilution of NaOAc, followed by adding 0.54 volume of cold isopropanol before observing the DNA precipitates. The solution was then spun down for another 2 min as previously mentioned.

The supernatant was discarded. The pellet was rinsed once with cold 70 % ethanol and dried on a paper towel. Fungal DNA was re-dissolved in TE (10 mM Tris-HCl, 0.1 mM EDTA pH 8.5) (Appendix 1) or in deionized distilled water. The final DNA concentration was adjusted in the range of 0.1-10 ng for a PCR reaction (147).

12.3 Polymerase chain reaction (PCR)

ITS1-5.8S-ITS2 regions of ribosomal DNA (rDNA) were amplified by PCR using the forward primer ITS5 and the reverse primer ITS4 as described by White et al. (79). Table 7 showed the primer sequences. Oligonucleotide primers were synthesized using ABI PRISM^{TM}, DNA/RNA synthesizer model 392, Perkin Elmer, by Bioservice Unit (BSU) at the National Science and Technology Development Agency (NSTDA), Bangkok, Thailand. The reaction mixture was prepared on ice. To accomplish the optimum PCR conditions, various concentrations of $MgCl_2$, *Taq* DNA polymerase, primers and template DNA were tested. This strategy was based on the standardized PCR protocols for taxonomic analysis. The amplification reaction was performed in the total volume of 50 µl: 0.2-5 ng of template DNA, 0.5 µM of each primer, 50 µM of individual dNTP, 2.5 nM of $MgCl_2$, 50 mM KCl, 10 mM of Tris-HCl at pH 8.3 and 0.5 U of *Taq* DNA polymerase (Appendix 1). For each test a primer

negative control was included without template DNA. Ice-cold PCR reaction tubes were transferred to an Eppendrof Mastercycler Gradient PCR machine.

The amplification process consisted of a pre-denaturation step at 95 °C for 3 min and 29 consecutive cycles of 95 °C for 50 sec (denaturation), 45 °C for 40 sec (annealing) and 72 °C for 40 sec (extension), with a final 72 °C for 10 min for extension in the last cycle.

Amplified products were analyzed by electrophoresis in 2 % agarose gel prepared in Tris-acetate-EDTA (TAE) buffer (Appendix 1) under 100 voltage of electrical power. The agarose gel was then stained in 0.5 μg/ml ethidium bromide solution and destained. The DNA was visualized under UV-transluminator at 312 nm. The molecular size of amplified products was determined by comparison with the standard marker λDNA digested with *Pst*I restriction enzyme.

Table 7. Primers for amplification of fungal (Pind 6) ribosomal RNA genes (78)

rRNA	Gene Primer [a]	Product Size (bp) [b]	T_m (°C) [c]
Nuclear, ITS			
ITS5	GGAAGTAAAAGTCGTAACAAGG		65
		315	
ITS2	GCTGCGTTCTTCATCGATGC		63
		290	
ITS3	GCATCGATGAAGAACGCAGC		62
		330	
ITS4	TCCTCCGCTTATTGATATGC		58

[a] All primers ITS5 and ITS3 are 5' primers; ITS2 and ITS 4 are 3' primers. Sequences are written 5'-3'.

[b] Product sizes are approximate based on the rRNA genes of *Saccharomyces cerevisiae*; the size of the region amplified is the product size minus the primers.

[c] T_m's were calculated by the method of Meinkoth and Whal (148).

13. DNA Sequencing and Phylogenetic Analyses

Purified PCR product was sequenced in an automated sequencer (ABI PRISM™ model 377, Perkin Elmer). Primers ITS2, ITS3, ITS4, and ITS5 (78) were used in the sequencing reactions in order to sequence both DNA strands. This was done by the Bioservice Unit (BSU) at the National Science and Technology Development Agency (NSTDA), Bangkok, Thailand.

The 5.8S sequence was used as a query sequence to search for similar sequences from GenBank. The most similar reference sequences were obtained. The ITS1 and ITS2 sequence of these reference sequences were used for subsequent phylogenetic analysis along with several reference sequences that showed high identity. The ITS1 and ITS2 sequences were aligned separately using Clustal W (149) and the results were adjusted manually to maximize the alignment. Phylogenetic analyses of data sets were done using the parsimony method of Phylip (150). Strengths of internal branches of resulting trees were statistically tested by bootstrap analysis for 100 replications.

CHAPTER IV

RESULTS

1. Medicinal Plant Samples and Endophytic Fungi Isolates

Five medicinal plant species (5 families) were collected from the forest area in Nakorn Ratchasima province on 12th, March 1999. A second collection was made in Chiangmai province on 12th July 2000 in which 2 plant species (2 families) were obtained. In the last collection, 29 plant species (20 families) were sampled from Songkhla province on 5th, December 2000, as shown in Table 8.

Culture of leaf-disc and limb sections yielded a wide variety of endophytic fungi in a every sample. A total of 123 morphologically distinct endophytic fungal isolates were selected for further study, as shown in Table 9.

Table 8. List of Thai medicinal plants used in this study.

Family	Scientific name	Thai name	Plant source
Anacardiaceae	*Spondias pinnata* Kurz	มะกอกป่า	Chiangmai
Annonaceae	*Uvaria rufa* Bl.	นมควาย	Songkhla

Family	Scientific name	Thai name	Plant source
Apocynaceae	*Alstonia macrophylla* Wall.	ทุ้งฟ้า	Songkhla
Apocynaceae	*Wrightia tomentosa* Roem. & Schult.	โมกมัน	Nakorn Ratchasima
Araceae	*Amorphophallus campanulatus* Bl. ex Decne.	บุกคางคก	Songkhla
	Homalomena aromatica Schott	เต่าเกียด	Songkhla
Araliaceae	*Trevesia palmata* Vis.	ต้างหลวง	Songkhla
Aristolochiaceae	*Aristolochia tagala* Cham.	กระเช้าผีมด	Chiangmai
Chloranthaceae	*Chloranthus inconspicuus* Sw.	เนียม	Songkhla
Compositae	*Crassocephalum crepidioides* S.Moore	ผักเผ็ดแม้ว	Songkhla
	Elephantopus scaber Linn.	โด่ไม่รู้ล้ม	Songkhla
	Mikania cordata Rob.	จี้ไก่ย่าน	Songkhla
	Pluchea indica Less.	ขลู่	Songkhla

Family	Scientific name	Thai name	Plant source
Ebenaceae	*Diospyros filipendula* Pierre ex Lecomte	ลำบิดดง	Songkhla
Euphorbiaceae	*Mallotus philippensis* Muell.Arg.	คำแสด	Songkhla
Malvaceae	*Urena lobata* Linn.	ขี้ครอก	Songkhla
Melastomataceae	*Melastoma malabathricum* Linn.	โคลงเคลง	Songkhla
	Arcangelisia flava Merr.	ขมิ้นเครือ	Songkhla
Menispermaceae	*Stephania hernandifolia* Walp.	ใบก้นปิด	Songkhla
	Tinospora crispa Miers ex Hook. f. & Thoms.	บรเพ็ด	Songkhla
Moraceae	*Streblus ilicifolius* Corner	ข่อยหนาม	Nakorn Ratchasima
Myrsinaceae	*Ardisia lanceolata* Roxb.	ราม	Songkhla
Myrtaceae	*Melaleuca leucadendra* Linn. var. *minor* Duthie	เสม็ดขาว	Songkhla
Rhamnaceae	*Ventilago denticulata* Willd.	รางแดง	Nakorn Ratchasima

Family	Scientific name	Thai name	Plant source
Rubiaceae	*Anthocephalus chinensis* Rich. ex Walp.	กระทุ่ม	Songkhla
	Ixora javanica DC.	เข็มทอง	Songkhla
Selaginellaceae	*Selaginella involuta* Spreng.	หญ้าร้องไห้	Songkhla
Simaroubaceae	*Eurycoma longifolia* Jack	ปลาไหลเผือก	Songkhla
Smilacaceae	*Smilax luzonensis* Presl	ยั้ง	Songkhla
Sonneratiaceae	*Sonneratia griffithii* Kurz	ลำแพนหิน	Songkhla
Taccaceae	*Tacca integrifolia* Ker-Gawl.	ค้างคาว	Songkhla
Verbenaceae	*Clerodendrum paniculatum* Linn.	พนมสวรรค์	Songkhla
	Stachytarpheta indica Vahl	พระอินทร์โปรย, พันงูเขียว	Songkhla
	Vitex peduncularis Wall. ex Schauer	กาสามปีก	Nakorn Ratchasima
Vitidaceae	*Tetrastigma campylocarpum* Planch.	ถลกบาตร	Nakorn Ratchasima

Table 9. Selected isolates of endophytic fungi obtained from Thai medicinal plant samples.

Plant host	Endophyte isolate	Plant section
Amorphophallus campanulatus Bl. ex Decne.	Acam 02 Acam 03 Acam 04 Acam 06	leaf
	Acam 08 Acam 09 Acam 11	limb
Anthocephalus chinensis Rich. ex Walp.	Achi 01 Achi 02	leaf
Ardisia lanceolata Roxb.	Alan 01	leaf
	Alan 02 Alan 06 Alan 07	limb
Alstonia macrophylla Wall.	Amac 02 Amac 04 Amac 05	leaf
	Amac 09	limb

Plant host	Endophyte isolate	Plant section
Arcangelisia flava Merr.	Afla 01	leaf
Aristolochia tagala Cham.	Atag 01	leaf
Crassocephalum crepidioides S. Moore	Ccre 01 Ccre 02 Ccre 03 Ccre 04	leaf
	Ccre 05 Ccre 06 Ccre 09	limb
Chloranthus inconspicuus Sw.	Cinc 03 Cinc 05	limb
Clerodendrum paniculatum Linn.	Cpan 03 Cpan 04 Cpan 05 Cpan 06	leaf
	Cpan 08 Cpan 09	limb
Diospyros filipendula Pierre ex Lecomte	Dfil 03 Dfil 05	leaf
	Dfil 08 Dfil 09	limb

Plant host	Endophyte isolate	Plant section
Eurycoma longifolia Jack	Elon 01 Elon 03	leaf
Elephantopus scaber Linn.	Esca 02 Esca 03 Esca 05	leaf
Homalomena aromatica Schott	Haro 03 Haro 05	limb
Ixora javanica DC.	Ijav 02 Ijav 04	leaf
	Ijav 07	limb
Mikania cordata Rob.	Mcor 01 Mcor 03 Mcor 04	leaf
	Mcor 05 Mcor 06 Mcor 07	limb
Melaleuca leucadendra Linn. var. *minor* Duthie	Mleu 01 Mleu 04 Mleu 05 Mleu 06	leaf

Plant host	Endophyte isolate	Plant section
Melastoma malabathricum Linn.	Mmal 01	leaf
	Mmal 03	limb
Mallotus philippensis Muell. Arg.	Mphi 02 Mphi 05 Mphi 06	leaf
	Mphi 09	limb
Pluchea indica Less.	Pind 04 Pind 05 Pind 06 Pind 07	limb
Sonneratia griffithii Kurz	Sgri 01 Sgri 03	leaf
	Sgri 06 Sgri 07 Sgri 08	limb
Stephania hernandifolia Walp.	Sher 01 Sher 03 Sher 04 Sher 05	leaf
	Sher 06	limb

Plant host	**Endophyte isolate**	**Plant section**
Streblus ilicifolius Corner	Sill 07	leaf
Stachytarpheta indica Vahl	Sind 01 Sind 02	leaf
	Sind 04 Sind 06 Sind 07	limb
Selaginella involuta Spreng.	Sinv 01 Sinv 02 Sinv 03	leaf
Smilax luzonensis Presl	Sluz 01 Sluz 02 Sluz 03	leaf
	Sluz 05	limb
Spondias pinnata Kurz	Spin 05	leaf
Tetrastigma campylocarpum Planch.	Tcam 12	limb

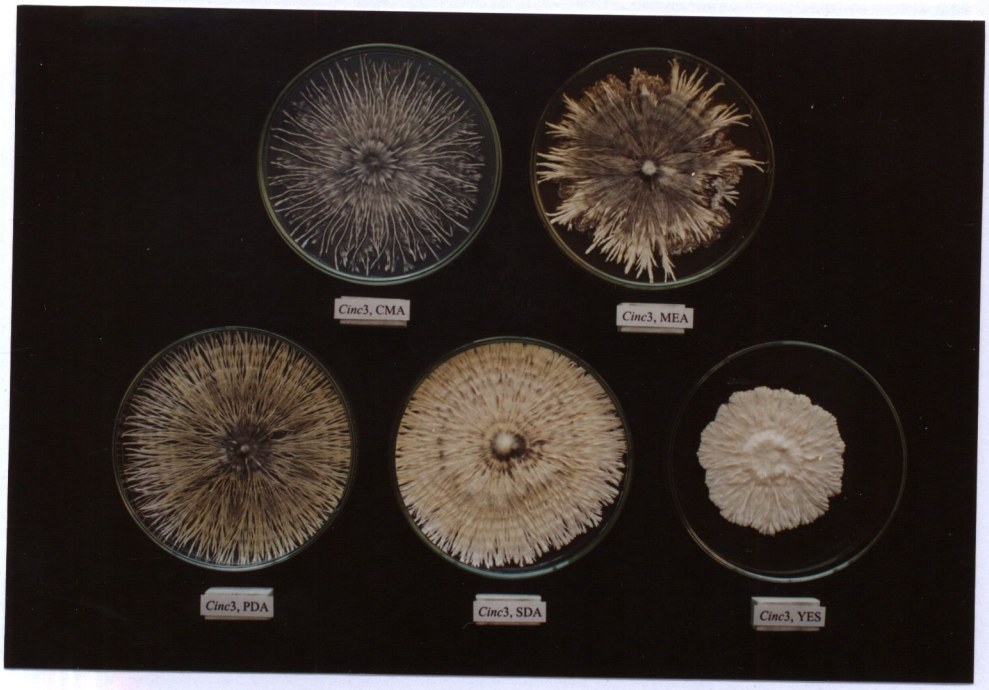
Plant host	Endophyte isolate	Plant section
Tinospora crispa Miers ex Hook. f. & Thoms.	Tcri 02 Tcri 04	leaf
	Tcri 06 Tcri 07 Tcri 08	limb
Tacca integrifolia Ker-Gawl	Tint 01 Tint 02 Tint 04	leaf
Trevesia palmata Vis.	Tpal 01 Tpal 05	leaf
Urena lobata Linn.	Ulob 01 Ulob 04	leaf
Uvaria rufa Bl.	Uruf 05	leaf
	Uruf 06 Uruf 08	limb
Ventilago denticulata Willd.	Vden 03 Vden 04	leaf
	Vden 07 Vden 08 Vden 10 Vden 11	limb

Plant host	Endophyte isolate	Plant section
Vitex peduncularis Wall. Ex Schauer	Vped 03 Vped 05 Vped 06 Vped 08	limb
Wrightia tomentosa Roem. Et Schult.	Wtom 01 Wtom 02 Wtom 03 Wtom 04 Wtom 05	limb

2. Characterization of Endophytic Fungi

Each fungus isolate was grown on five different culture media, *i.e.* CMA, MEA, PDA, SDA, and YES, for 14 days at room temperature. Most of the fungal endophyte isolates did not produce conidia or spores on these common mycological media. Colonial morphology of 10 fungal isolates is shown in Figures 12-21, as examples. Because the lack of sporulation in most of the cultures, identification by conventional morphological-based methods could not be made. However, each isolate showed unique colonial morphology allowing them to be considered distinctive fungi. Thus, an isolate code, based on the scientific name of the host plant, was given to each fungus for identification purposes.

A



B

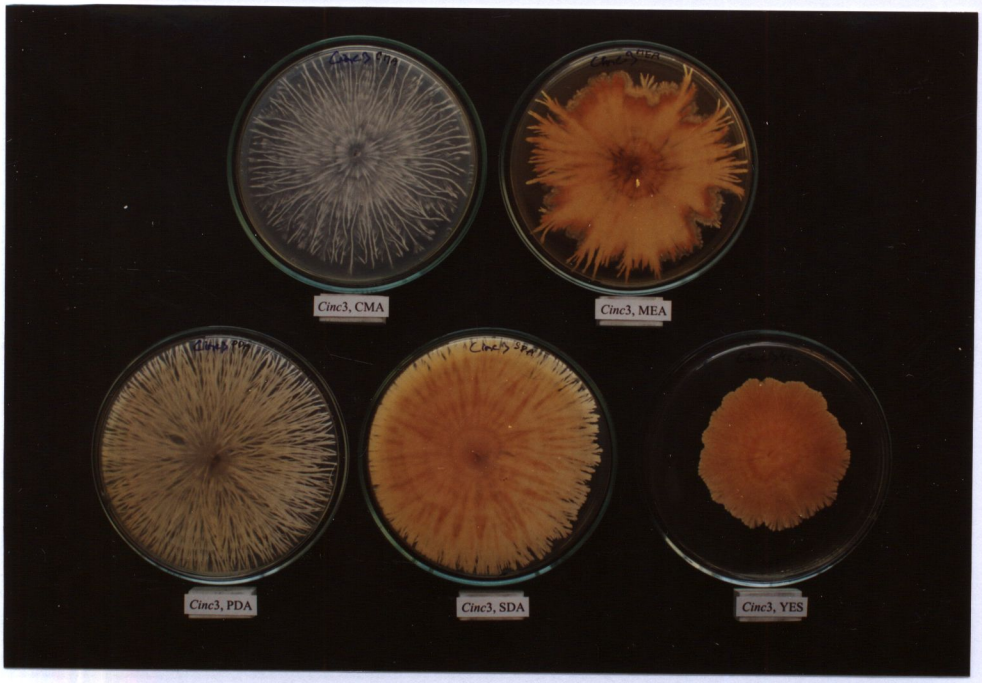


Figure 12. Colonial morphology of endophytic fungus isolate, Cinc 3, on five different media (CMA, MEA, PDA, SDA, and YES) after cultivation for 14 days at room temperature. Appearance on the obverse side **(A)**, and on the reverse side **(B)**.

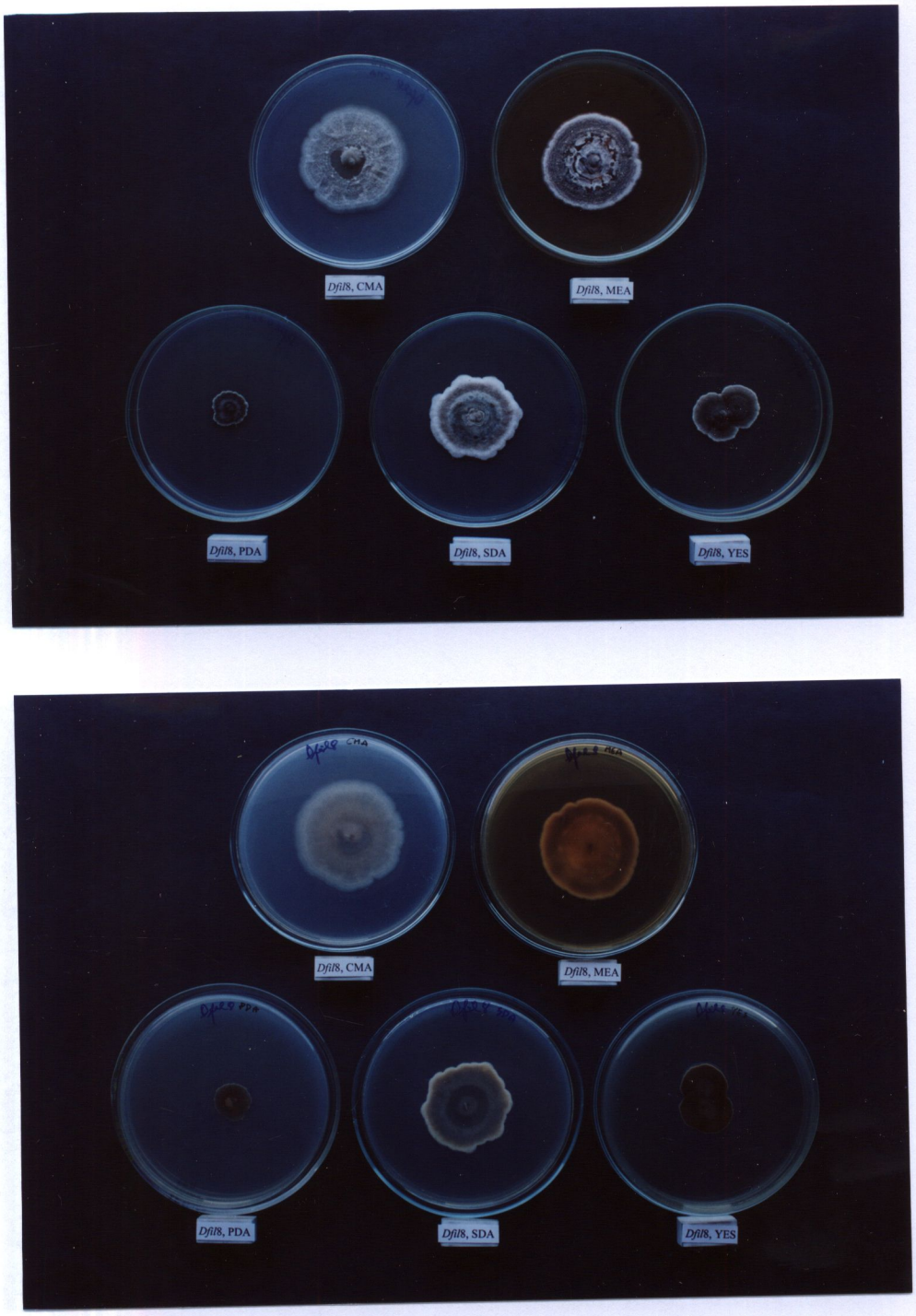


Figure 13. Colonial morphology of endophytic fungus isolate, Dfil 8, on five different media (CMA, MEA, PDA, SDA, and YES) after cultivation for 14 days at room temperature. Appearance on the obverse side **(A)**, and on the reverse side **(B)**.

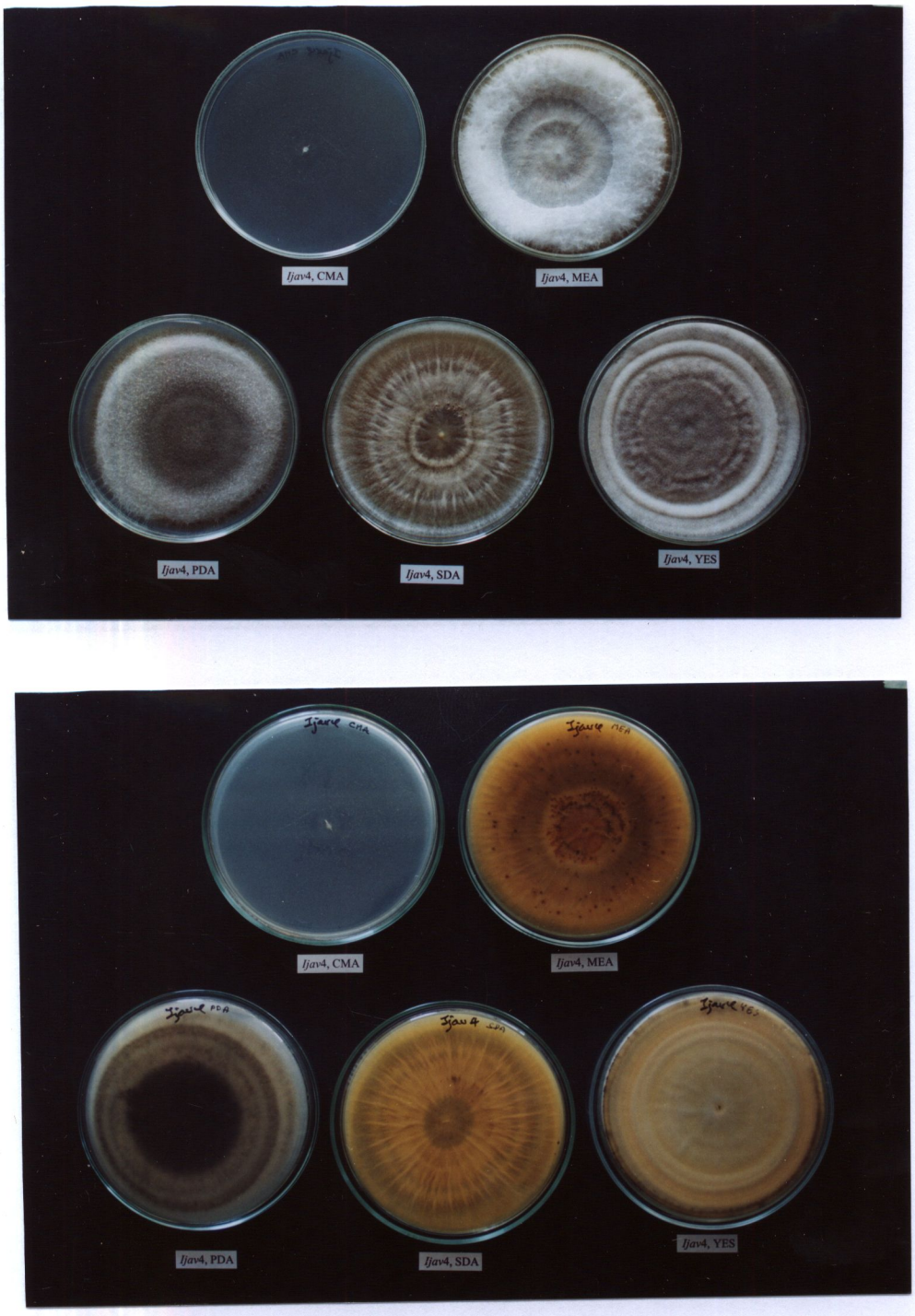
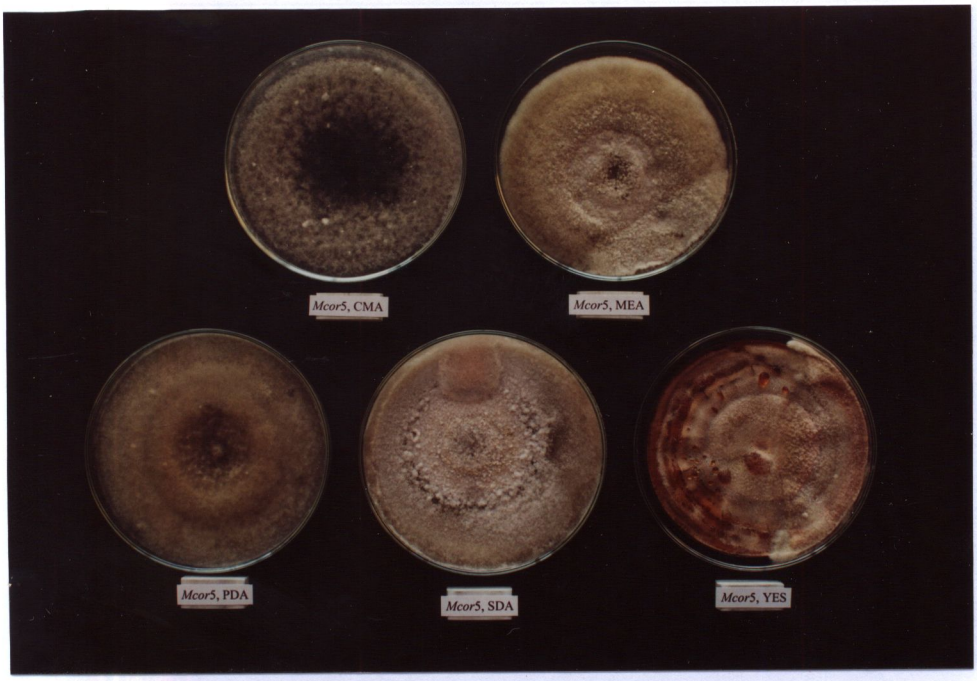


Figure 14. Colonial morphology of endophytic fungus isolate, Ijav 4, on five different media (CMA, MEA, PDA, SDA, and YES) after cultivation for 14 days at room temperature. Appearance on the obverse side **(A)**, and on the reverse side **(B)**.

A



B

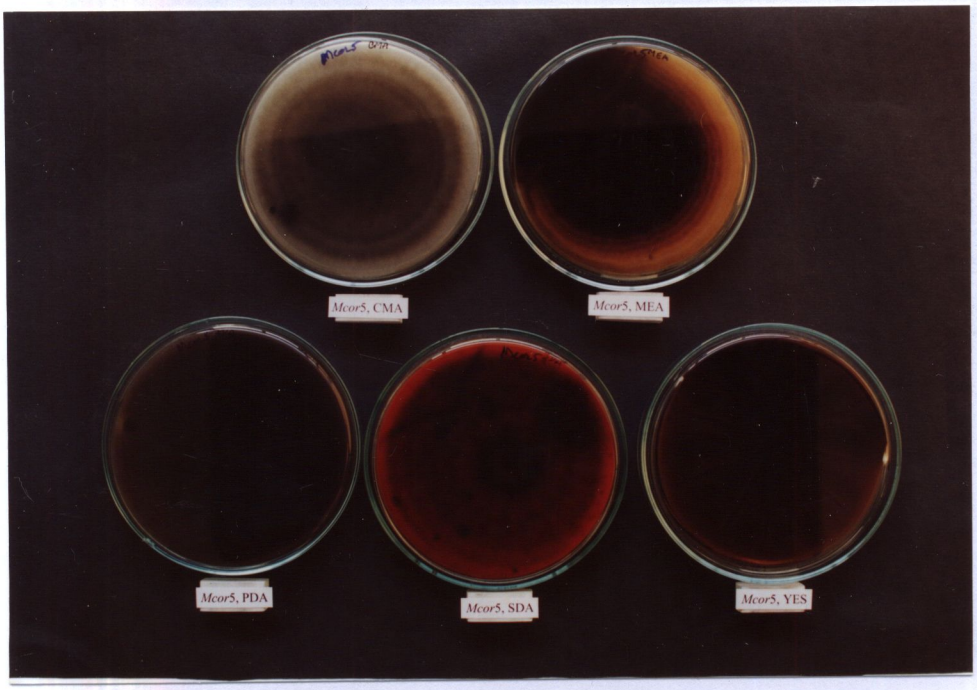


Figure 15. Colonial morphology of endophytic fungus isolate, Mcor 5, on five different media (CMA, MEA, PDA, SDA, and YES) after cultivation for 14 days at room temperature. Appearance on the obverse side **(A)**, and on the reverse side **(B)**.

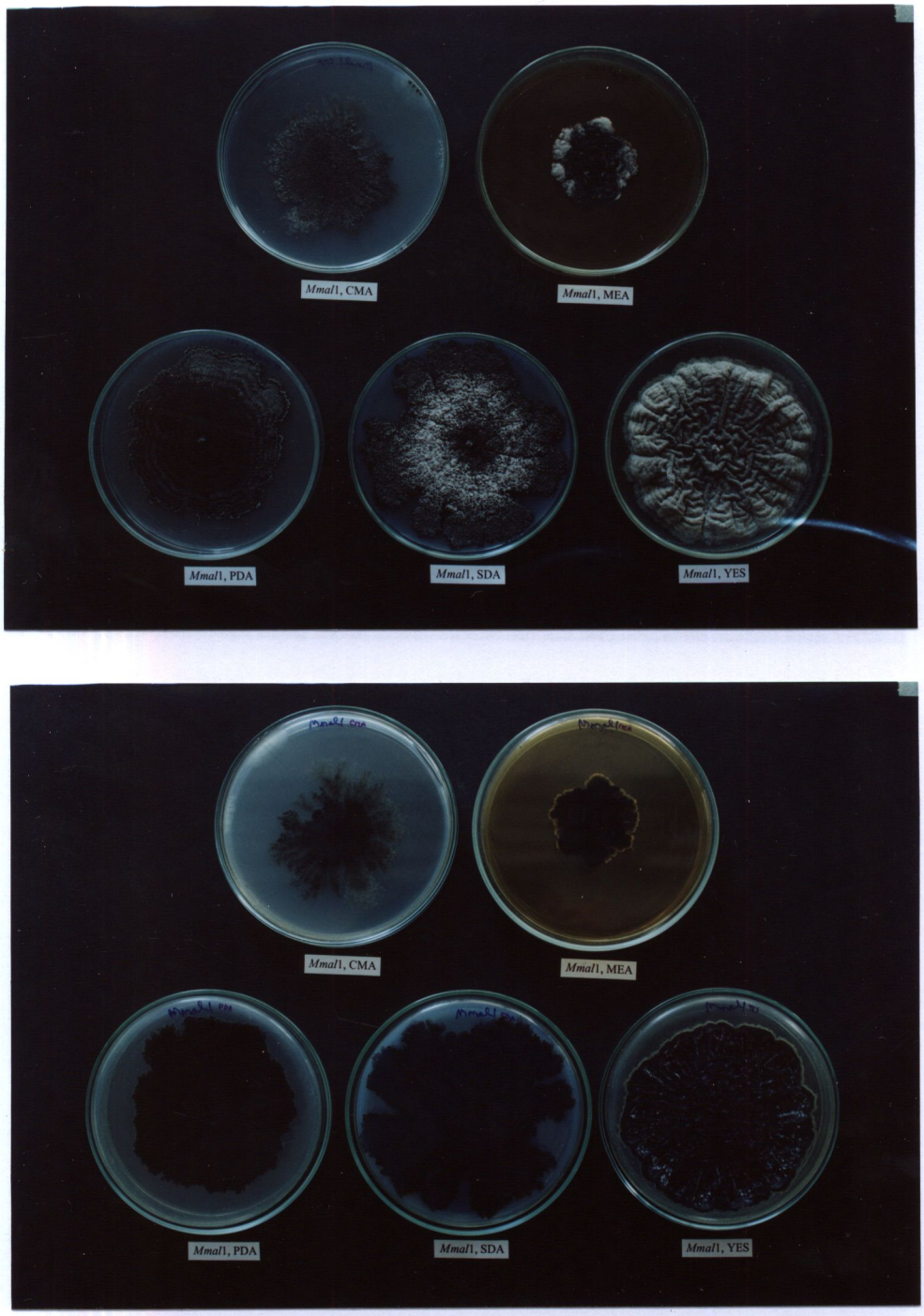


Figure 16. Colonial morphology of endophytic fungus isolate, Mmal 1, on five different media (CMA, MEA, PDA, SDA, and YES) after cultivation for 14 days at room temperature. Appearance on the obverse side **(A)**, and on the reverse side **(B)**.

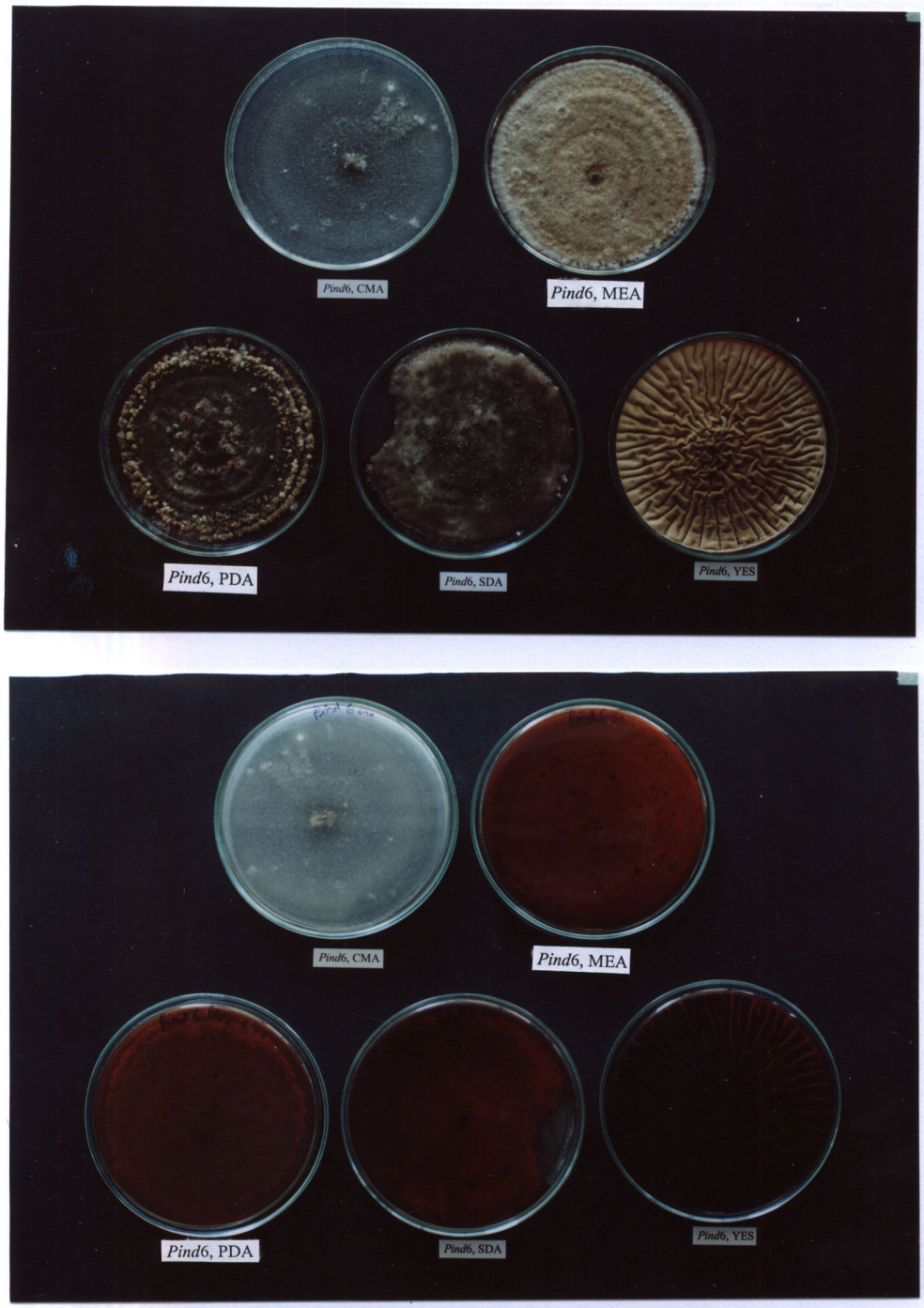


Figure 17. Colonial morphology of endophytic fungus isolate Pind 6, on five different media (CMA, MEA, PDA, SDA, and YES) after cultivation for 14 days at room temperature. Appearance on the obverse side **(A)**, and on the reverse side **(B)**.

A

B

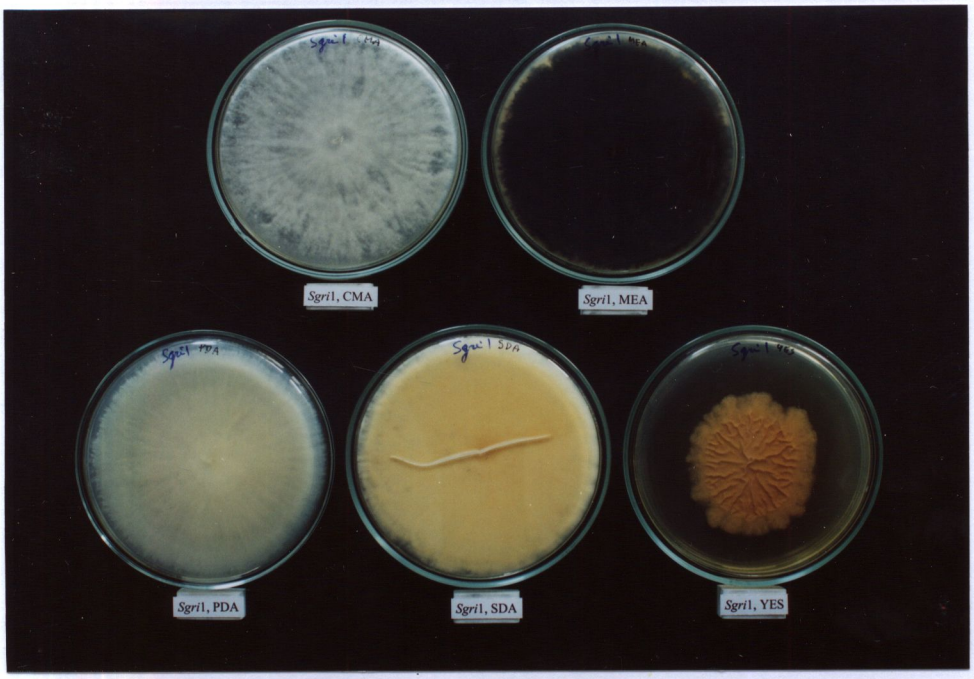
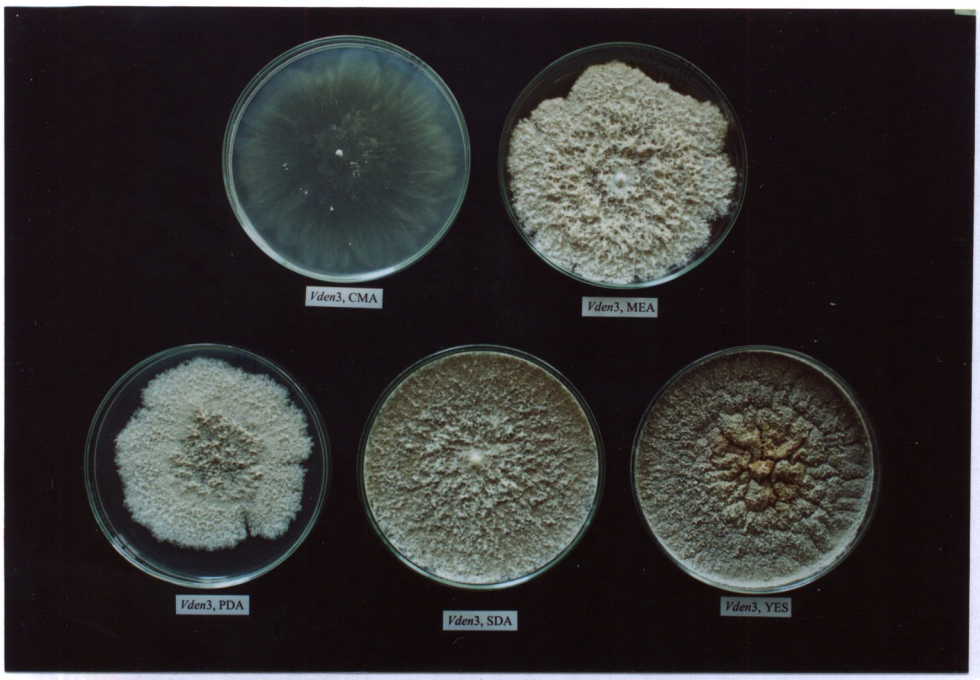


Figure 18. Colonial morphology of endophytic fungus isolate Sgri 1, on five different media (CMA, MEA, PDA, SDA, and YES) after cultivation for 14 days at room temperature. Appearance on the obverse side **(A)**, and on the reverse side **(B)**.

Figure 19. Colonial morphology of endophytic fungus isolate, Tpal 1, on five different media (CMA, MEA, PDA, SDA, and YES) after cultivation for 14 days at room temperature. Appearance on the obverse side **(A)**, and on the reverse side **(B)**.

A



B

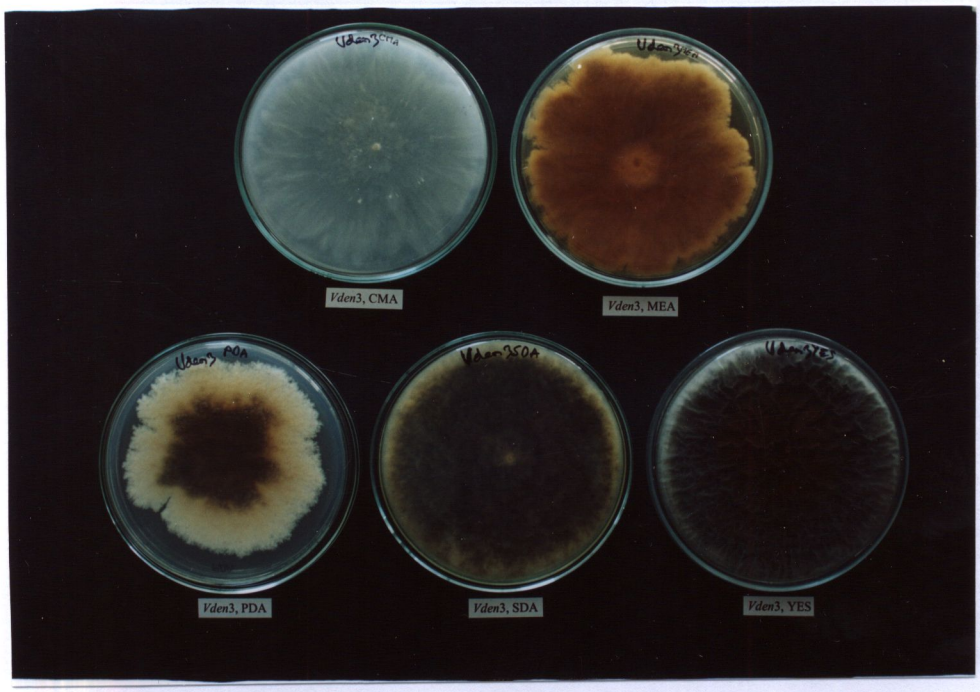


Figure 20. Colonial morphology of endophytic fungus isolate, Vden 3, on five different media (CMA, MEA, PDA, SDA, and YES) after cultivation for 14 days at room temperature. Appearance on the obverse side (**A**), and on the reverse side **(B)**.

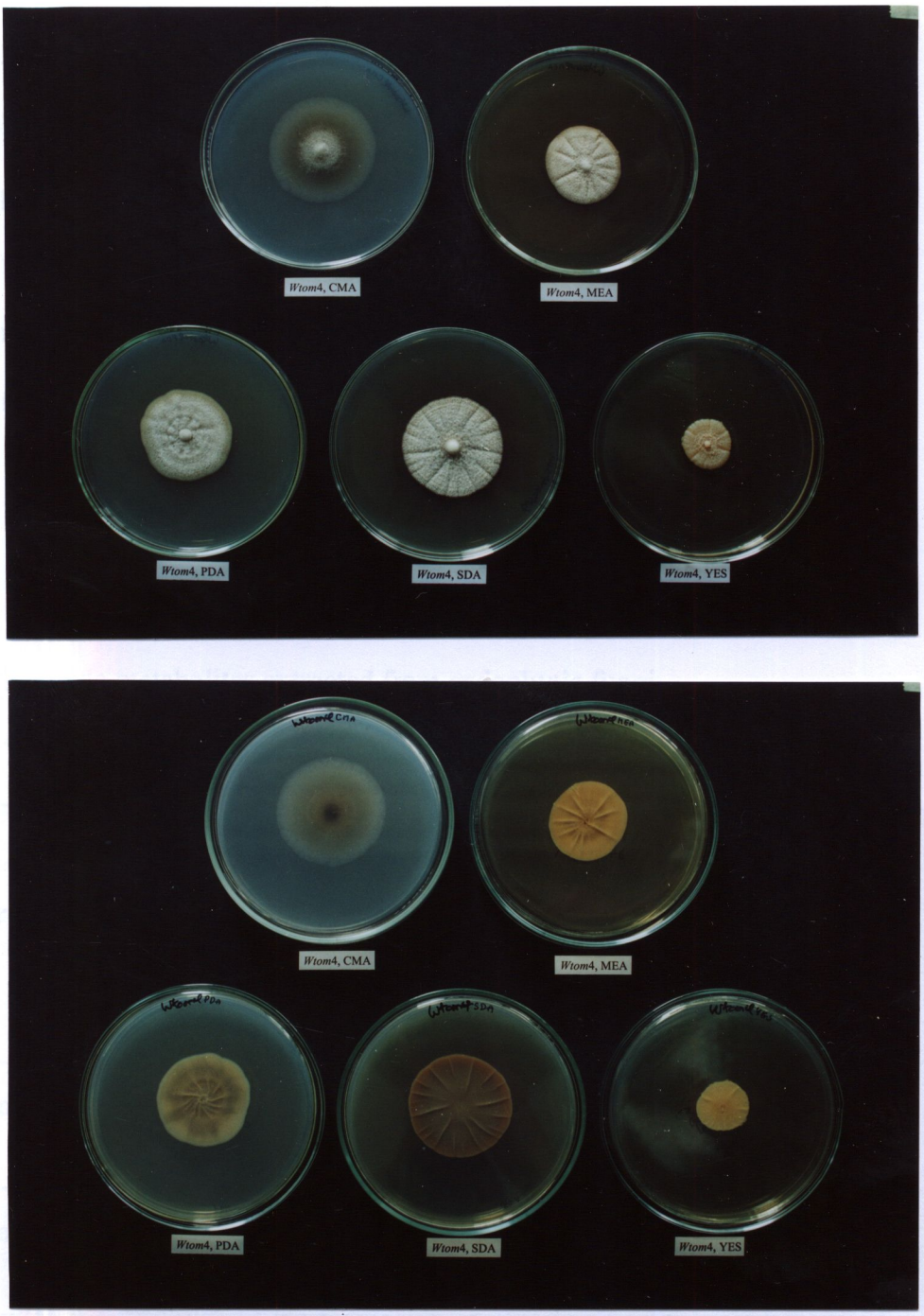


Figure 21. Colonial morphology of endophytic fungus isolate, Wtom 4, on five different media (CMA, MEA, PDA, SDA, and YES) after cultivation for 14 days at room temperature. Appearance on the obverse side **(A)**, and on the reverse side **(B)**.

3. Crude Metabolites from Endophytic Fungi

The 123 selected isolates of endophytic fungi were grown in MCz and YES broth. Mycelial mass was separated from culture broth by filtration and each part was extracted separately to obtain crude metabolite extracts of the mycelium and that of the broth. From the cultures of 123 fungi in 2 type of media, a total of 478 crude extract samples were obtained as shown in Table 10.The crude extracts were further tested for antibacterial, antifungal, antimycobacterium, and antimalarial activity.

Table 10. The crude metabolites extracted from endophytic fungi.

Culture media	**Number of crude extract**	
	Culture broth	**Mycelium**
MCz medium	120	119
YES medium	120	119

4. Viable Count of Standardized Inoculum

Plate counts was performed for standardized inocula whose turbidity matched a 0.5 McFarland standard. The CFU/ml values shown in Table 11 were means calculated from triplicate determinations.

Table 11. Colony forming unit (CFU) /ml of standardized inoculum with turbidity matched to 0.5 McFarland standard ($OD_{625} = 0.1 \pm 0.03$).

Test microorganisms	CFU/ml
S. aureus	7.1×10^7
E. faecalis	5.3×10^7
B. subtilis	1.2×10^7
E. coli	5.6×10^7
P. aeruginosa	4.4×10^7
C. albicans	8.1×10^5
T. mentagrophytes	2.0×10^6

5. Antibacterial and Antifungal Activity by INT Microdilution Assay

Figure 22A shows an example of an INT microdilution plate assay of anti-*S. aureus* while Figure 22B shows an example an anti-*C. albicans* plate assay. The INT is an oxidation-reduction (redox) indicator dye for detecting growth of microorganisms. Microbial growth causes the redox indicator to change from a colorless oxidized form to a reduced violet form. Violet wells in Figure 22A and 22B are those where growth of test microorganisms occurred and colorless wells are those where growth inhibition was caused by extracts of the fungal endophytes.

Results of antimicrobial activity assays against *S. aureus*, *E. faecalis*, *B. subtilis*, *E. coli*, and *P. aeruginosa*, and antifungal activity assays against, *C. albicans*, and *T. mentagrophytes* are summarized in histograms in Figure 23.

For activity against *S. aureus* from MCz medium, 46 isolates showed activity with culture broth extracts only while 2 isolates showed activity with mycelial extracts only and 12 isolates showed activity with both. For YES medium, anti-*S. aureus* activity was found only in the culture broth of 42 isolates.

For anti-*E. faecalis* activity from MCz medium, 40 isolates showed activity with culture broth extracts only, 3 with mycelial extracts only and 9 with both. With YES medium, 44 isolates were active with broth extracts only, one with mycelial extract only and 5 with both.

For anti-*B. subtilis* activity from MCz medium, 94 isolates showed activity with broth extracts only, 7 with mycelial extracts only and 39 with both. From YES medium, 92 isolates showed activities from culture broth extract only 7 with mycelial extracts only and 28 with both.

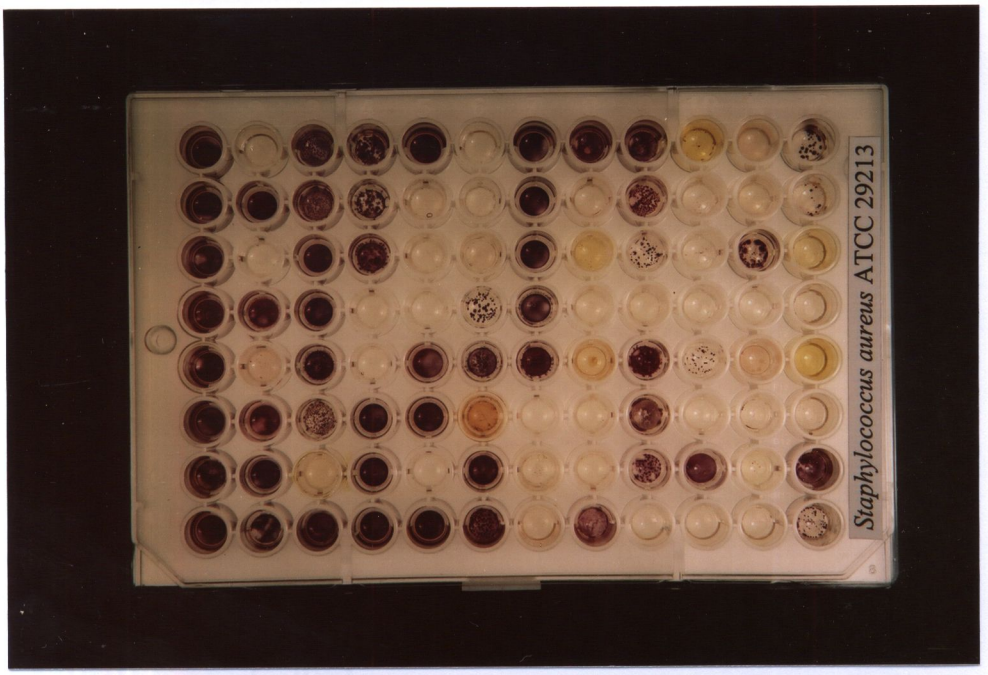
Anti-*E. coli* activity was found in culture broth extracts only for 8 isolates in MCz medium and 11 isolates in YES medium.

Anti-*P. aeruginosa* activity was found in culture broth extracts only for 3 endophyte isolates in both MCz and YES medium.

Anti-*C. albicans* activity was found in culture broth extracts only for 26 isolates cultured in MCz medium and 19 isolates cultured in YES medium.

For anti-*T. mentagrophytes* activity was shown for 63 isolates from MCz medium with culture broth extracts only 8 isolates with mycelial extracts only and 1 isolate with both. From YES medium, 72 isolates showed activity with culture broth extracts only and 1 isolate showed activity with mycelial extract only.

A



B

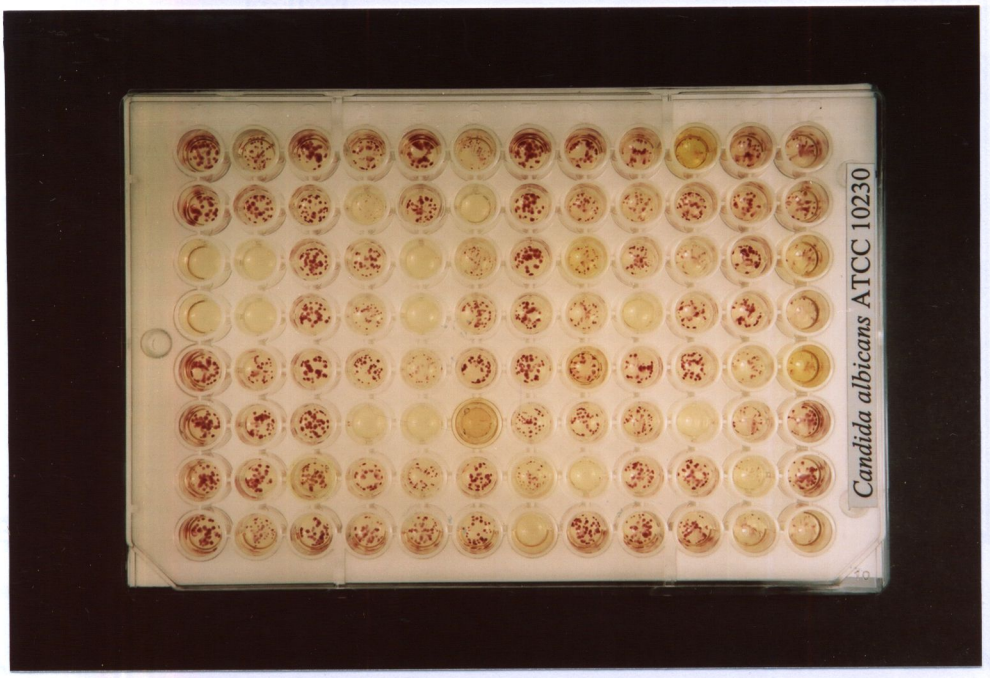


Figure 22. INT assay for antimicrobial activity. **A**; INT microdilution assay of anti-*S. aureus* activity. **B**; INT microdilution assay of anti-*C. albicans* activity. Violet wells indicate microbial growth and clear or colorless wells indicate growth inhibition.

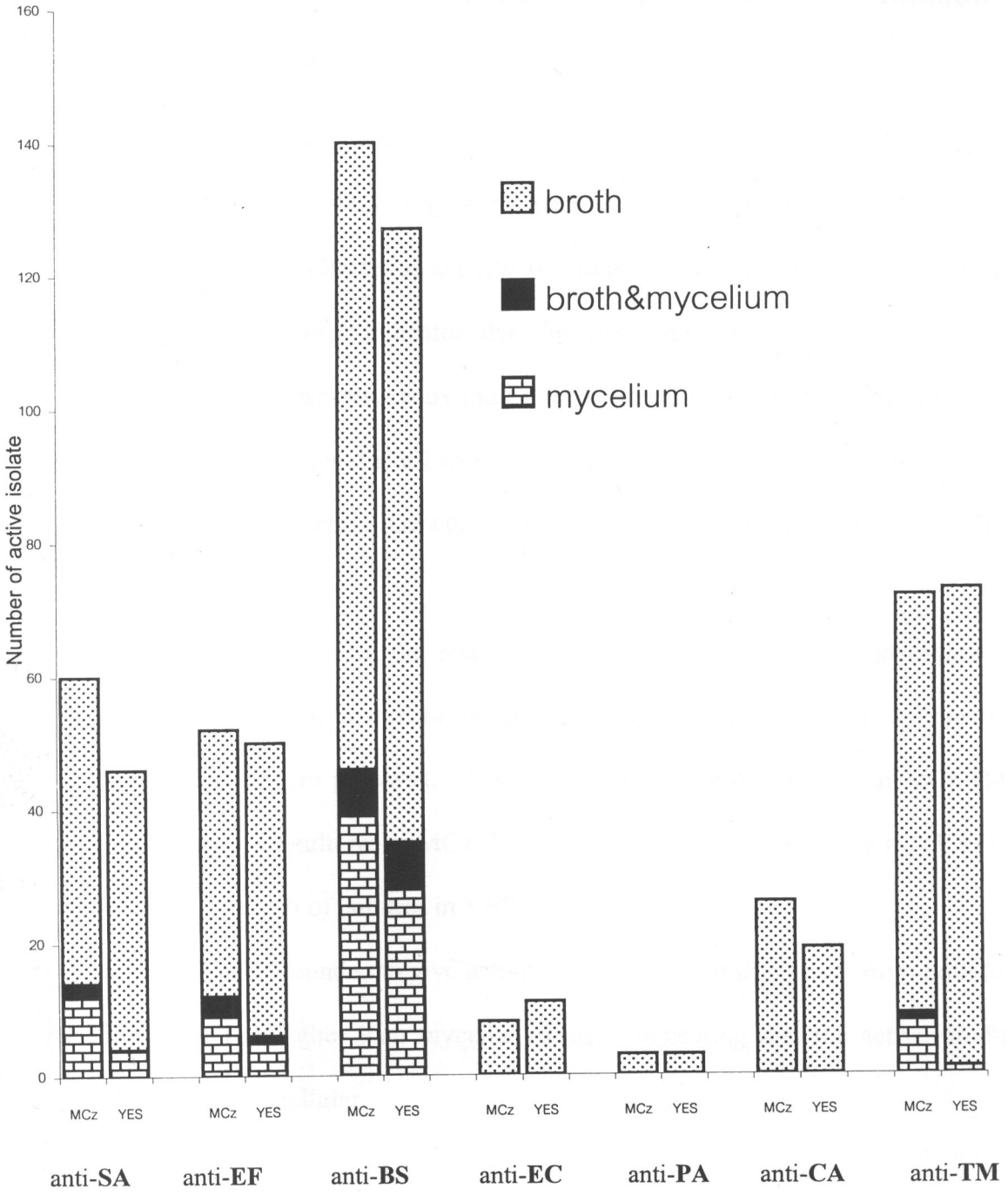


Figure 23. A summary of INT microdilution assay results for antibacterial activity of endophyte extracts. Bacteria tested were *S. aureus;* SA, *E. faecalis;* EF, *B. subtilis;* BS, *E. coli;* EC, and *P. aeruginosa;* PA and fungi tested were *C. albicans*; CA, and *T. mentagrophytes*; TM by microdilution assay.

6. Anti-*M. tuberculosis* Activities of Crude Extracts of Endophytic Fungi Cultures

Anti-*M. tuberculosis* activities was assayed by the Alamar-blue microdilution method (MABA) as shown in Figure 24. Microdilution plates with 96 wells were used to determine antimycobacterial activity of crude extracts at various concentrations. Alamar-blue is a redox indicator dye for detecting growth of *M. tuberculosis*. Microbial growth causes the redox indicator to change from a blue oxidized form to a reduced red form. Red wells in Figure 24 are growth of *M. tuberculosis* occurred and blue wells are those where growth inhibition was caused by extracts of the fungal endophytes.

Table 12 summarizes the results of activity tests against *M. tuberculosis* by ranking from highest to lowest minimum inhibitory concentration (MIC). Four types of crude extracts were prepared. These were from broth of cultures in MCz (MCz broth), mycelium of cultures in MCz (MCz mycelium), broth of cultures in YES (YES broth), and mycelium of cultures in YES (YES broth).

Most isolates founded to give activity with culture broth extracts from both MCz and YES medium rather than mycelial extracts, indicating that the active products founded to be extracellular.

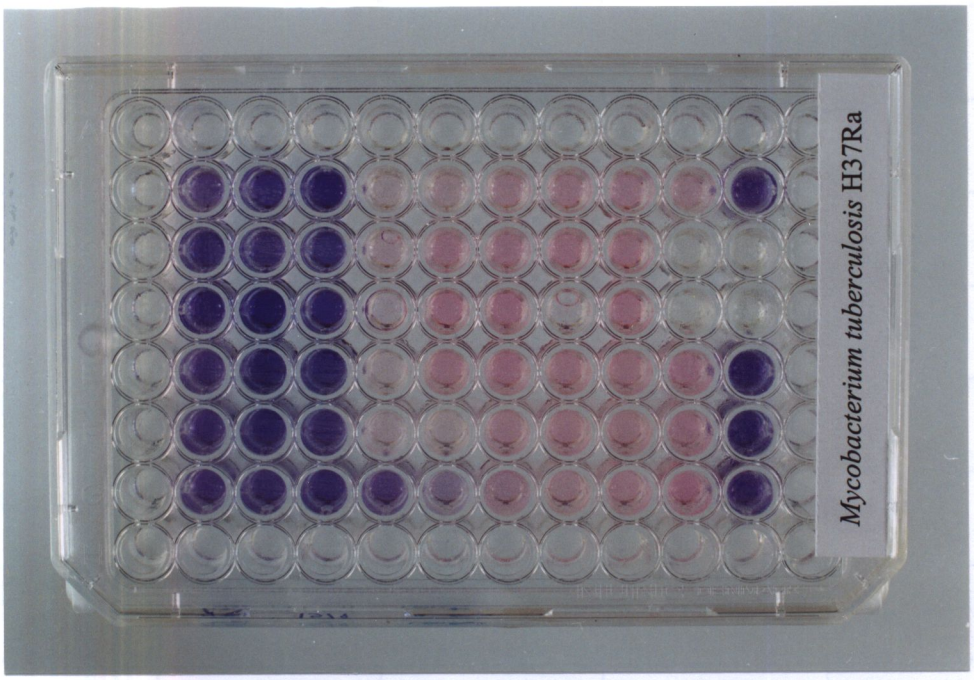


Figure 24. Example of an Alamar-blue microdilution method (MABA) test for anti-TB (*M. tuberculosis*) activity. Red wells indicated microbial growth while blue wells indicated growth inhibition.

Table 12. Anti TB (*M. tuberculosis*) activities as determined by Alamar-blue microdilution plate assay (MABA).

Minimum Inhibitory Concentration (μg/ml)	**Number of crude extracts that showed anti TB activity**			
	MCz broth	**MCz mycelium**	**YES broth**	**YES mycelium**
MIC=15.63	0	0	1	0
MIC=31.25	1	0	2	0
MIC=62.5	2	0	4	0
MIC=125	4	0	3	0
MIC=250	32	2	33	2
MIC=500	33	1	36	2
MIC>500	23	2	22	2
Inactive	26	115	19	113

7. Biological Activity of Endophyte Isolates for Antimalarial Activity

7.1 Growth and development of *P. falciparum* from *in vitro* culture

Growth and development of the malarial parasite *P. falciparum* in continuous culture were observed in thin blood smears with Field's stain. Cultures of *P. falciparum* K1 were carried out in 96-well microdilution plates. From primary culture, the parasite started with the first ring form stage in and developed to young trophozoites, trophozoites, schizonts, and then mature schizonts containing merozoites. Merozoites were released from infected red blood cells (RBCs) and then invaded new RBCs as is shown in Figure 25A. They then began a new cycle of erythrocytic schizogony within 48 h.

The ring form of *P. falciparum* measured about 1/5 the diameter of a RBC (Figure 25B). In the Field's stain examination, the ring form of parasite comprised a densely stained violet nucleus connected to a circular, violet ring that surrounded the food vacuole. The rings developed into the trophozoite stage (Figure 25C) by an increase in cytoplasm to occupy approximately 1/3 of RBC within 18-24 hours. It stained violet for chromatin in nuclei, dark blue for condensed strips of cytoplasm and brown for pigment granules. Next, immature schizonts and mature schizonts developed (Figure 25D) during 18-24 h of incubation. Immature schizonts could be identified by nuclear changes associated with the beginning of cell division. These were visible as large RBC inclusions with violet nuclei and slightly reddish cytoplasm almost completely filling the RBC nuclei. Mature schizonts were visible within 30-36 h, and yielded approximately 16-24 merozoites (Figure 25E). These appeared as large RBC inclusions with violet nuclei and deep red cytoplasm, completely filling in RBC.

The merozoites were then released from RBC to invade new RBC (Figure 25F), and re-initiate a cycle of erythrocytic schizogony.

7.2 *In vitro* morphological changes of *P. falciparum* caused by crude extracts of endophytes

Based on morphological changes in *in vitro* malarial screening cultures, it was evident that some extracts were effective against the malarial parasite. The assays employed extracts at concentrations between 500 μg/ml to 125 μg/ml over an incubation period of 24-48 h. Anti malarial activity was determined by examination of thin blood smears using Field's stains with treated and untreated (control) blood cells. In control RPMI 1640 complete medium for 48 h., numbers increased 4-5 fold from the initial number at the time zero and wells showed all stages of healthy parasites, *i. e.* rings, trophozoites, and schizonts (Figure 25B, 25C and, 25D).

Examples of results from anti-malarial crude extracts are shown in Figure 26. Some dead ring stages appeared as only small violet stained chromatin dots without cytoplasm (Figure 26A) while others showed abnormal cytoplasm with vacuoles. The RBC containing dead rings was normal in size and shape. With dead trophozoites, the chromatin dot or nucleus was violet, the vacuole was colorless or translucent, and the cytoplasm was wrinkle or absents (Figure.26B).

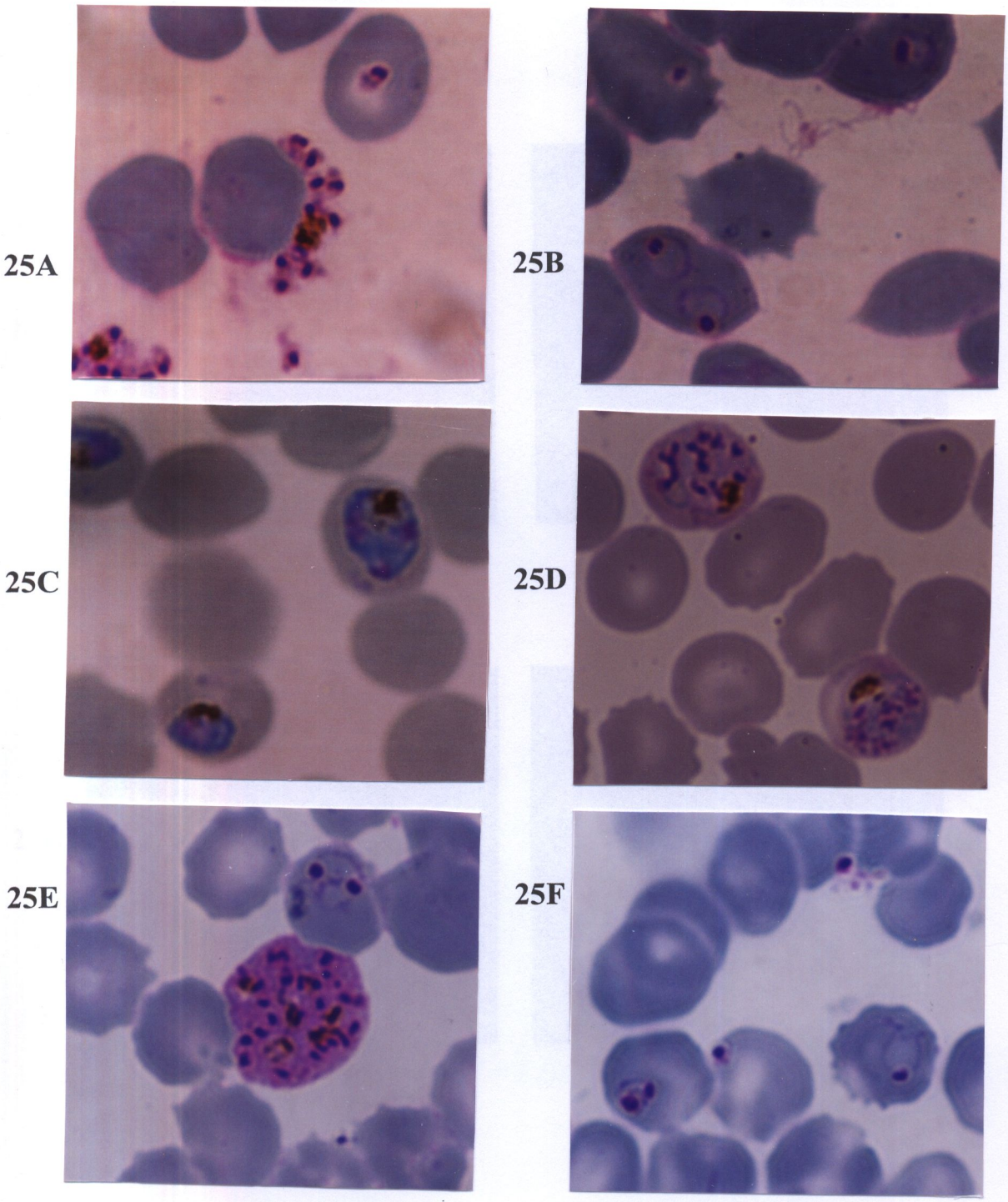
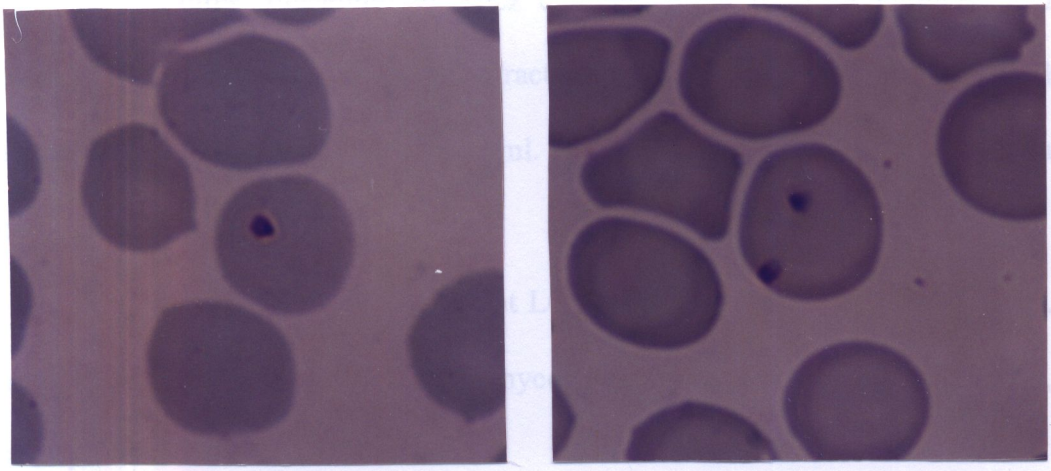


Figure 25. Growth and development of *P. falciparum* from *in vitro* culture. **25A:** Merozoites stage, **25B:** Ring stage, **25C:** Trophozoite stage, **25D:** Schizont stage, **25E:** Mature schizont stage, and **25F:** Merozoites invading new RBCs.

26A



26B

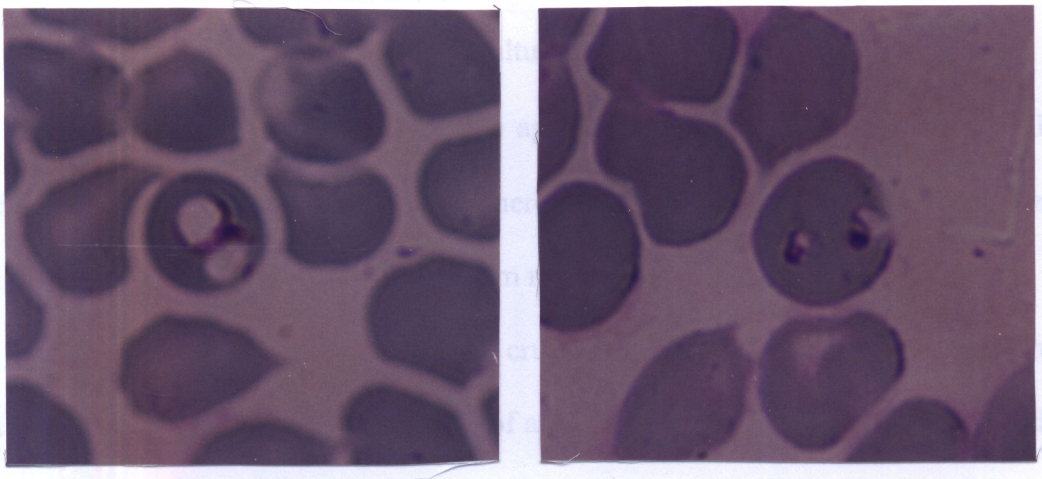


Figure 26. *In vitro* morphological changes of *P. falciparum* as the result of exposure to crude extracts from endophytes. **26A:** dead ring stages, **26B:** dead trophozoites.

7.3 Anti-malarial activities against *P. falciparum*

As before, the four types of crude metabolite extracts were those from broth of MCz cultures, mycelium of MCz cultures, broth of YES cultures, and mycelium of YES cultures. The crude extracts were tested at three affluent lethal concentrations (LC); 500, 250, and 125 μg/ml. The result are shown as histograms in Figure 27.

For anti-*P. falciparum* activity at LC 500 μg/ml were found in 29, 9, 39, and 9 samples extracts of MCz broth, MCz mycelium, YES broth, and YES mycelium, respectively.

The number of active metabolites against *P. falciparum* at LC 250 μg/ml were 19, 20, 3, and 4 samples from culture broth extract of MCz, and YES cultures, and mycelium extract from MCz and YES culture, respectively.

The highly effective metabolites against *P. falciparum* at LC 150 μg/ml were found only in culture broth extract. There were found in 18 sample from MCz medium, while 14 samples from YES medium respectively.

Most of activities were found in crude extracts from culture broth of both media (MCz and YES), indicated that most of anti-malarial metabolites were produced from endophytic fungi dissolved into liquid cultures.

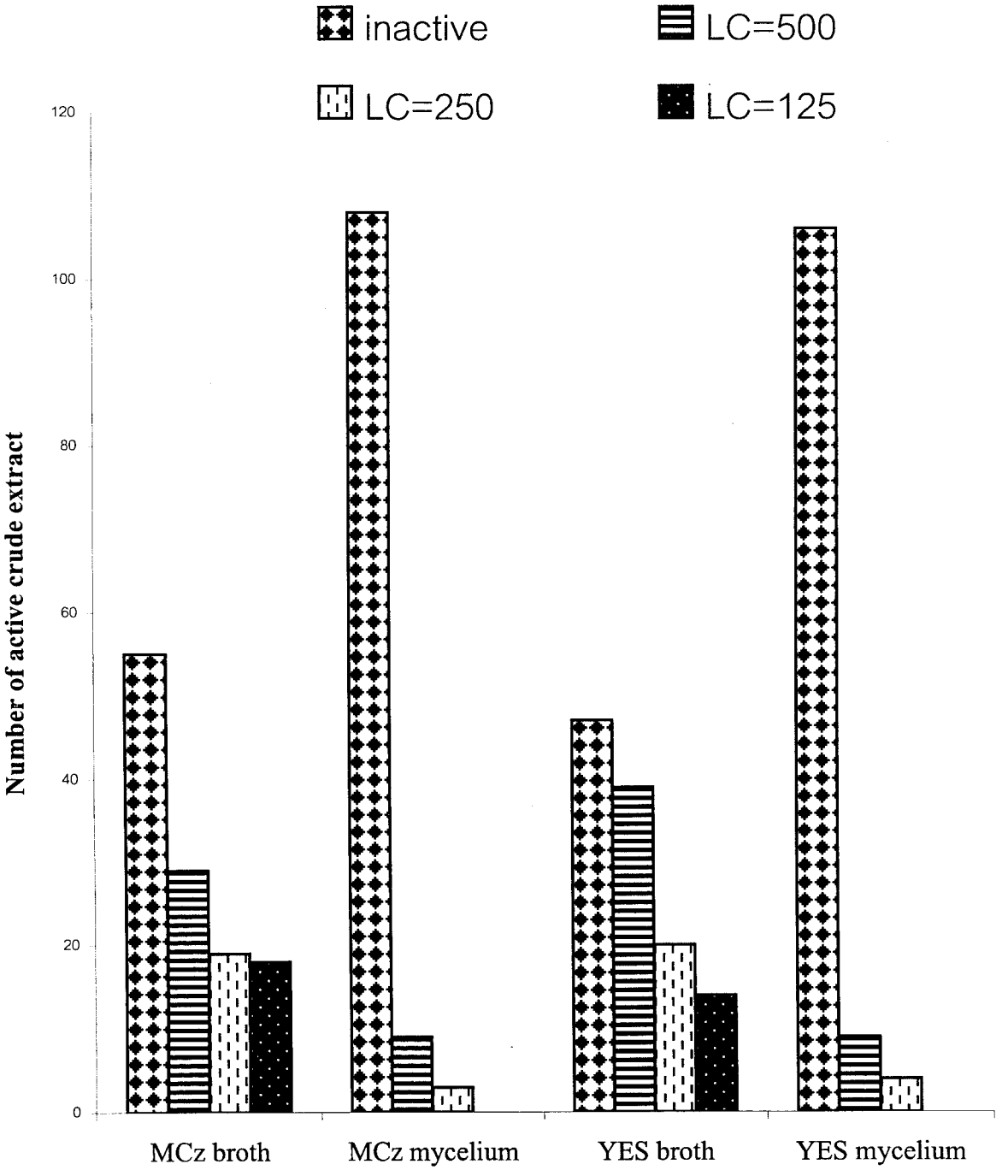


Figure 27. Anti-malarial activities of fungal endophyte crude extracts.

8. Ribosomal DNA Sequence of Endophytic Fungi

8.1 The PCR product of ITS1, 5.8S and ITS2 regions of ribosomal DNA

PCR conditions were optimized to amplify a rDNA gene within the selected isolate, Pind 6. A pair of primers ITS4 and ITS5 (78) was used to amplify a DNA fragment at the region of ITS1, 5.8S and ITS2 of rDNA. Figure 28 shows the PCR product for 25-amplification cycles by 2 % agarose gel electrophoresis. The optimization condition was previously described in the Materials and Methods section. The size of this product was compare with the molecular marker of λ*Pst*I. The product lane 2 in Figure 28 was a single band found in between the 514 bp to 805 bp marker bands.

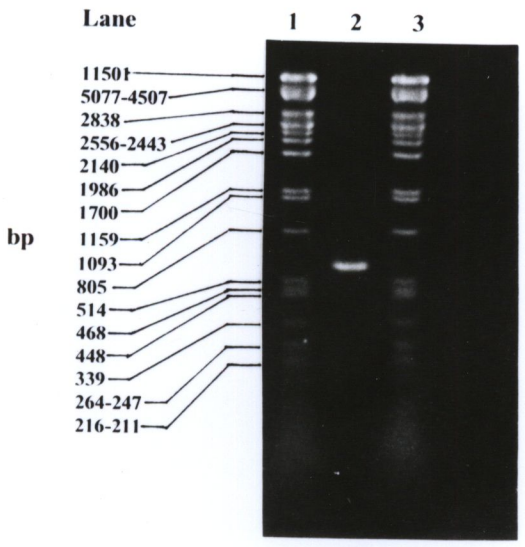


Figure 28. Agarose gel electrophoresis analysis of the PCR product from amplification of ITS1, 5.8S and ITS2 regions of rDNA. **Lane 1** and **3** were the standard size marker (λ*Pst*I), and **Lane 2** was the PCR product of Pind 6.

8.2 Nucleotide sequence of partial 18S and 28S sequences and complete ITS1-5.8S-ITS2 sequence of isolate Pind 6

Sequencing of the PCR product resulted in a 590 bp fragment. This comprised partial of the 18S sequence, complete ITS1-5.5S-ITS2 sequences and partial of the 28S sequence, as shown in Figure 29.

1	TGGAA	GTAAA	AGTCG	TAACA	AGGTC	TCCGT	TGGTG	AACCA	40
			18S	**ITS1**					
41	GCGGA	GGGAT	CATTG	CTGGA	ACGCG	CCCCA	GGCGC	ACCCA	80
81	GAAAC	CCTTT	GTGAA	CTCAT	ACCTA	TCGTT	GCCTC	GGCGC	120
121	TAGCT	GGCCT	CTTAG	GCCCC	TTGGA	GACAA	GGAGC	GGCGC	160
161	GCCGG	CGGCC	CACTC	AACTC	TGTTT	CTCTG	TGAAT	CTCTG	200
					5.8S				
201	AGTAA	AAAAC	AAATG	AATCA	AAACT	TTCAA	CAACG	GATCT	240
241	CTTGG	TTCTG	GCATC	GATGA	AGAAC	GCAGC	GAAAT	GCGAT	280
281	AAGTA	ATGTG	AATTG	CAGAA	TTCAG	TGAAT	CATCG	AATCT	320
321	TTGAA	CGCAC	ATTGC	GCCCT	CTGGC	ATTCC	GGAGG	GCATG	360
						ITS2			
361	GCATG	CCTGT	TCGAG	CGTCA	TTTCA	ACCCT	CAAGC	CTGGC	400
401	TTGGT	GCTGG	GGCGC	TGCTT	CTCAC	AGGGG	CAGGC	CCTGA	440
441	AATTC	AGTGG	CGGGC	TCGCC	TCCGA	GCGCA	GTAGT	ACATC	480
481	TCGTT	CTGGA	AGGCC	TGGCG	GTGCC	CTGCC	GTTAA	ACCCC	520
				28S					
521	AACTC	TGAAA	ATTTG	ACCTC	GGATC	AGGTA	GGAAT	ACCCG	560
561	CTGAA	CTTAA	GCATA	TCAAT	AAGCG	GAGGA			

Figure 29. Nucleotide sequence of the ITS1, 5.8S and ITS2 regions of rDNA of isolate Pind 6.

8.3 Phylogenetic analyses

The search for similar sequences to 5.8s rRNA sequence of Pind 6 resulted in 4 reference taxa with 100 % identity. They were *Phyllosticta spinarrum*, *Phyllosticta* sp. IF 03312, *Botryosphaeria vaccinii* and *Guignardia philoprina*. Another 17 taxa showed 99 % (157/158) identity.

Alignment of ITS1 and ITS2 sequences of isolate Pind 6 with those of reference taxa resulted in sequence of 190 sites (141 informative) and 185 sites (99 informative), respectively.

The phylogenetic relationship inferred from these data is shown in Figure 30 and 31, respectively. These inferred phylogenetic trees were consensus trees generated from 690 parsimonous trees for ITS1 data and 1812 parsimonous trees for ITS2 data. Two major clades were obtained and supported by 100% bootstrap value in both trees. Pind 6 was found to be in the clade of *Phomopsis* and its teleomorph, *Diaporthe*.

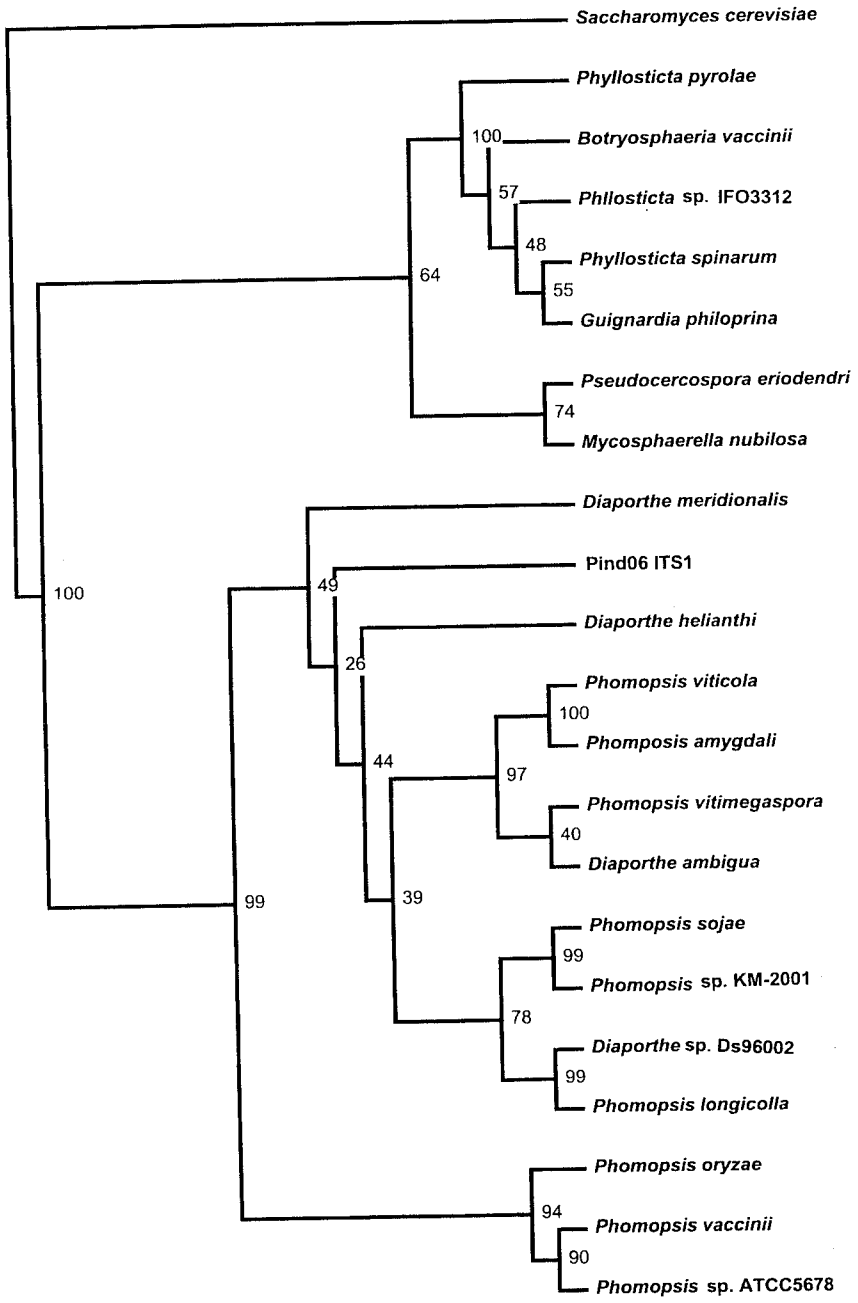


Figure 30. Consensus parsimonous tree inferred from the analysis of ITS1 sequences of isolate Pind 6 and reference taxa. *Saccharomyces cerevisiae* was used as the outgroup. The numbers at nodes indicate the bootstrap value from 100 replications

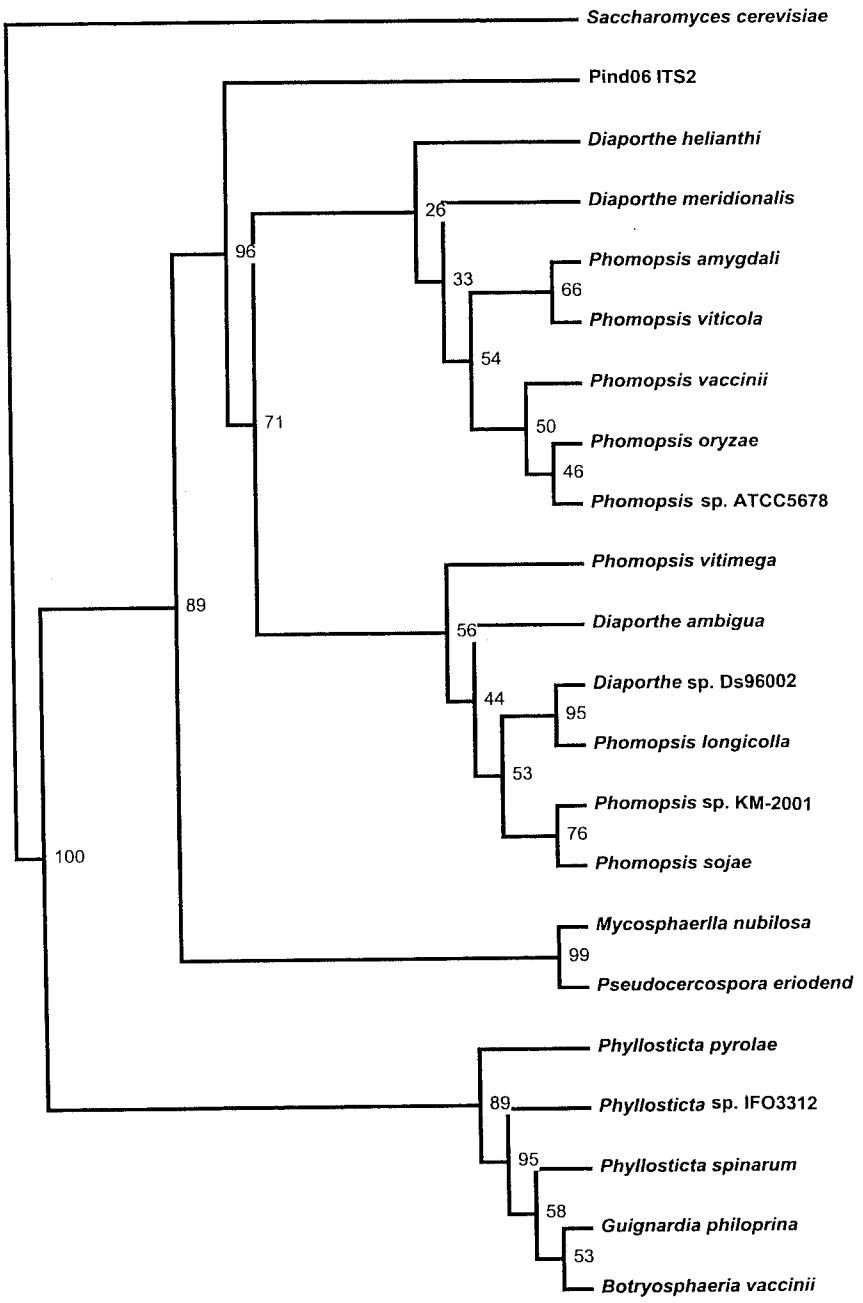


Figure 31. Consensus parsimonous tree inferred from the analysis of ITS2 sequences of isolate Pind 6 and reference taxa. *Saccharomyces cerevisiae* was used as the outgroup. The numbers at nodes indicate the bootstrap value from 100 replications

CHAPTER V

DISCUSSION

1. Occurrence and Diversity of Endophytic Fungi in Thai Plants

Thai medicinal plants have been used for healing purposes since ancient times. In the modern days, substantial research work has been done to study phytochemical compositions and biological or especially pharmacological properties of extracts or pure compounds isolated from these plants. However, the existence of fungal endophytes in Thai medicinal plants has never been examined nor the biological potentials of such fungi investigated.

Thailand is situated in a region of tropical rain forests that host a large proportion of the world's plant species as well as other living species. A large number of biologically active metabolites are probably produced in these ecosystems. Given the large number and varieties of plant species, the numbers of their associated fungal endophyte must also be enormous.

It was evident from this study that endophytic fungi could be isolated from every plant sample both from leaves and limbs. The isolated fungi exhibited diverse morphological and cultural characteristics indicating a high degree of diversity. By inference it could be postulated that other groups of Thai plants, would also host a large numbers of fungal endophytes with highly diversified genetics and properties.

2. Fungal Endophyte Isolation Technique

Surface-sterilization with sodium hypochlorite and 70 % ethyl alcohol followed by hyphal tip isolation was used to obtain microbes from the plant specimens after place on Tap water agar (TWA). TWA contains no nutrients and would not support microbial growth but provided a humid environment. The fungi that grew out from the plant segments must therefore have grown out from the plant tissue and used it as nutrient and could therefore called endophytic fungi. TWA had the advantage that out growing hyphae showed up clearly and could be easily isolated by cutting of hyphal tips.

The population of endophytes that could be isolated may have been dependent on plant sample storage. It was found that endophytes could not be obtained from plant samples kept too long in the refrigerator. During transportation and storage, the plant samples need to be kept moist and tissue death must be prevented by aeration to maintain respiration. Overhumid conditions must also be avoided to present growth of epiphytic fungi and bacteria. Selective media may help to increase the diversity of endophytes recovered from leaves or twigs.

3. Microdilution Assays and Bioactive Metabolite Screening Strategies

The method for antibacterial and antifungal testing was performed by microdilution methods modified from Woods *et al.* (136) and Espinel-Inggoff *et al.* (137) following NCCLS recommendations. The procedures for determining antimicrobial activity were carried out by broth-based methods. The microtiter trays contained several fungal crude metabolites to be tested simultaneously. Microbial inocula were standardized by dilution and adjusted to a final volume of 100 CFU/well. The *p*-iodonitrotetrazolium violet (INT) oxidation-reduction color indicator used for

detecting growth (violet color) and growth inhibition (colorless) gave high sensitivity and could be rapidly applied to many extracts derived from only a small amount of fungal culture medium. After addition of 10 µl of 2 mM INT solution, color changes could be observed within 30 min for bacterial cultures, and 1 h for yeast cultures. Some fungal crude extracts were strongly colored and interfered with the color of INT formazan. In such cases, the number of viable cells remaining in the microtiter well after incubation with fungal metabolites could be determined by the plate count method. The INT method was not applicable for testing inhibitory activity against filamentous fungi because color changes could hardly be observed. In this case, direct observation of mycelial growth inhibition or retardation using an inverted microscope was preferred.

Testing of antimycobacterium activity followed the method of Collins & Franzblau (138) using a microdilution technique. The colorimetric, Alamar blue oxidation-reduction dye was the indicator of cellular growth by visual measurement. This assay was successful for rapid, low-cost, and high-throughput for new drug screening against the slow growing *M. tuberculosis* (138).

The antimalarial assay was determined by microscopic measurement following methods of the World Health Organization (WHO) in 1990 and based on a morphologic end point. The ability of crude extracts to prevent transition from ring to schizont stages over a 24-h incubation period (151) was determined after Field's staining. In the procedure, *Plasmodium falciparum* K1 strain resistant to chloroquine and pyrimethamine (152) but sensitive to artemisinin (quinqhausu) was used.

By testing in this way, it was assumed that metabolites active against *P. falciparum* K1 might have a better chance of acting against the parasite by a mechanism(s) different from those of chloroquine and pyrimethamine. In addition,

metabolites being active against ring and trophozoite stages should not be related to artemisinin, which is active against the schizont stage (152). Such metabolites might be potentially novel compounds.

4. Biological Activities of Extracts from Endophytic Fungi

Based on the biological activities summarized in Table 13, the number of active extracts obtained from culture broth of both MCz and YES was significantly higher than the number obtained from their respective mycelial extracts.

Thus, the bioactive metabolites were mostly produced and secreted into the extracellular fluid. Perhaps this may explain the biological role of endophytic fungi in their host plants. They may survive in the plants as symbionts and provide protective substances that can accumulate in plant tissues to inhibit or kill invading pathogens.

Most of the fungal isolates showed activity against *B. subtilis* and more than 50 % produced active metabolites against the dermatophyte, *T. mentagrophytes*. Anti-*P. aeruginosa* activity was relatively rare as it was observed in only four fungal isolates or 2.5 % of total tested. These isolates should be studied further. They may produce strongly active compounds.

Table 13. Summary of biological activities of endophytic fungi.

Crude metabolite	Percentage of isolated endophytes producing potential bioactive compounds*								
	SA[a]	EF[a]	BS[a]	EC[a]	PA[a]	CA[a]	TM[a]	TB[b]	PF[c]
MCz broth	38.3	33.3	78.3	6.7	2.5	21.7	52.5	27.5	24.2
MCz mycelium	10.1	7.6	32.8	0.0	0.0	0.0	6.7	0.8	7.7
YES broth	35.0	36.7	76.7	9.2	2.5	15.8	60.0	30.0	32.5
YES mycelium	3.4	3.4	23.5.	0.0	0.0	0.0	0.8	1.7	7.6

* **SA**; anti-*Staphylococcus aureus*, **EF**; anti-*Enterococcus faecalis*, **BS**; anti-*Bacillus subtilis*, **EC**; anti-*Escherichia coli*, **PA**; anti-*Pseudomonas aeruginosa*, **CA**; anti-*Candida albicans*, **TM**; anti-*Trichophyton mentagrophytes*, **TB**; anti-*Mycobacterium tuberculosis*, **PF**; anti-*Plasmodium falciparum.*

[a] Active crude extract at MIC 1000-5000 μg/ml.

[b] Active crude extract at MIC 500 μg/ml.

[c] Active crude extract at LC 500 μg/ml.

5. Ribosomal DNA Sequence of Endophytic fungi

Because most of endophytic fungi are sterile, nucleotide sequences of rRNA genes provide an attractive approach in their taxonomy. The nucleotide sequence of the 5.8S DNA region is conserved and can be used to delineate species relationships (153). In this study, the nucleotide sequence of the 5.8S DNA gene of endophytic fungus isolate Pind 6 showed 99.37-100 % identity to the sequences of fungi in class Dothideomycetes, *Phomopsis* and its teleomorph, *Diaporthe*. The ITS DNA region is much more variable and can be used to separate taxa from classes to genera (153). In order to delineate isolate Pind 6 below the class level, nucleotide sequence of its ITS region was determined and aligned with ITS regions of reference taxa. Phylogenetic trees inferred from ITS1 and ITS2 data showed that Pind 6 was evolutionarily close to *Phomopsis* and *Diaporthe* (Figure 30 & 31). However, Pind 6 could not be assigned to any known species in these genera recorded in GenBank. This was for 2 reasons. First, the bootstrap value of branch in the inferred tree was very low. Second, its branch in the inferred tree diverged from the *Phomopsis* and *Diaporthe* clade.

6. Endophytic Fungi may Relate to Fungi in Plant Disease

This study shows that endophytic fungus Pind 6 could be a new species of *Phomopsis* and *Diaporthe* whose several members were known to be plant pathogens. *D. meridionalis* was known to cause stem canker in soybean (154), *D. helianthi* causes brown stem canker in sunflower (155) and *D. ambigua* is a fruit tree pathogen (156).

P. viticola causes dead arm in vines (157), *P. amygdali* causes sunken canker in peach (158, 159), *P. vitimegaspora* causes leaf lesions in vines(159), *P. sojae* and *P. longicolla* cause pod blight in soybean(159, 160), and *P vaccinii* causes twig blight in cranberry(161). These evidences of plant pathogenic nature of its close relatives suggested that the Pind 6 fungus might live as a latent infection within its host plant *Pluchea indica* Less.

CHAPTER VI

CONCLUSION

This work has shown that a large number of endophytic fungi can be isolated from Thai medicinal plants. It opens up the possibility that some of these plants may derive their medicinal properties as a direct or indirect result of such endophytes. In addition, few of the fungi isolated could be identified to genus or species due to the lack of sporulation structures, and one of such isolates proved to have a rDNA sequence that did not match with any known fungus in GenBank database. This further suggests that many of the endophytes in Thai medicinal plants, and probably in other known or undescribed Thai plants may be special presently unknown to science. As such, the potential for producing novel metabolites is further increased.

This preliminary study has shown that endophytes from Thai medicinal plants can produced extracellular compounds with activities against bacterial, fungal and protozoa pathogens of man and these extracts should be studied further so that the active ingredients can be identified.

The natural roles of the active compounds shown in this study remain to be established. It is possible that they may provide some protection from pathogens of their host plants as part of mutualistic association.

REFERENCES

1. Hawksworth DL. The fungal dimension of biodiversity: magnitude, significance, and conversation. Mycol Res 1991; 95: 641-55.

2. Strobel GA, Long DM. Endophytic microbes embody pharmaceutical potential. ASM News 1998; 64 (4): 263-8.

3. Dreyfuss MM, Chapela IH. Potential of fungi in the discovery of novel, lowmolecular weight pharmaceuticals. In: Gullo VP, editor. The discovery of natural products with therapeutic potential. Boston, MA: Butterworth-Heinemann; 1994; 49-80.

4. Hyde KD, Hawksworth DL. Measuring and monitoring the biodiversity of microfungi. In: Hyde KD, editor. Biodiversity of tropical microfungi. Hong Kong: Hong Kong Uni Press; 1997; 11-28.

5. Fesher PJ, Petrini O. Fungal saprobes and pathogens as endophytes of rice (*Oryza sativa* L). New Phytol 1992; 137-43.

6. Petrini O. Fungal endophytes of tree. In: Andrews JH, Hirano SS, editors. Microbial ecology of leaves. New York: Springer-Verlag; 1991; 179-97.

7. Siegal MR, Latch GCM, Johnson MC. Fungal endophytes of grasses. Annu Rev Phytopathol 1987; 25: 293-315.

8. Clay K. Fungal endophytes, grasses and herbivores. In: Barbosa P, Krischik VA, Jones CG, editors. Microbial mediation of plant of plant-herbivore interactions. New York: John Wiley&Sons Inc; 1991; 199-252.

9. Lewis DH. Concept in fungal nutrient and the origin of biotrophy. Biol Rev 1973; 48: 261-78.

10. Lewis DH. Micro- organisms and plants: The evolution of parasitism and mutualism. In: Carlile MJ, Skehel JJ, editors. Symposium of the society for general microbiology 24th. Cambridge: Cambridge Uni Press; 1974; 367-92.

11. Petrini O. Taxonomy of endophytic fungi of aerial plant tissues. In: Fokkema NJ, van den Huvel J, editors. Microbiology of the phyllosphere. Cambridge: Cambridge Uni Press; 1986; 175-87.

12. Sinclair JB, Cerkauskas RF. Latent infection vs endophytic colonization by fungi. In: Redlin SC, Carris LM, editors. Endophytic fungi in grasses and woody plants. Minnesota: APS Press; 1996; 3-29.

13. Freeman S, Rodriguez RJ. Genetic conversion of a fungal plant pathogen to a nonpathogenic, endophyte mutualist. Science 1993; 260:75.

14. Cerkauskas RF. Latent colonization by *Colletotrichum* spp. : epidermologygical considerations and implications for mycoherbicides. Can J Plant Patho 1988; 10: 297-310.

15. Cekauska RF, Dhingra OD, Sinclair JB, Asmus G. *Amaranthus spinosus*, *Leonotis nepetaefolia*, and *Leonurus sibricus*: new hosts of *Phomosis* spp. in Brazil. Plant Dis 1983; 67: 821-24.

16. Hartman GL, Manandhar JB, Sinclair JB. Incidence of *Colletotrichum* spp. on soybeans and weeds in Illinois and pathogenicity of *Colletotrichum truncatum*. Plant Dis 1986; 70: 780-2.

17. Bacon CW. Isolation, culture, and maintenance of endophytic fungi of grasses. In: Labeda DP, editor. Isolation of biotechnological organism from nature. New York: McGram-Hill Publishing Co; 1990; 259-82.

18. Boddy L, Griffith GS. Role of endophytes and latent invasion in the development of decay communities in sapwood of angiospermous trees. Sydowia 1989; 41: 41-73.

19. Bernstein ME, Carroll GC. Internal fungi in old-growth Douglas fir foliage. Can J Bot 1977; 55: 644-53.

20. Baird RE. Mycobiota of bark associated with seven strains of Cryphonectria prasitica on two hardwood tree sprcies. Mycotaxon 1991; 40: 23-33.

21. Dreyfuss MM, Petrini O. Further investigations on the occurrence and distribution of endophytic fungi in tropical plants. Bot Helv 1984; 94: 33-40.

22. Petrini O, Dreyfuss MM. Endophytische pilze in epiphytischen Araceae, Bromiliaceae und Orchidaceae. Sydowia 1981; 34: 135-48.

23. Bill GF. Isolation and analysis of endophytic fungal communities from woody plants. In: Redlin SC, Carris LM, editors. Endophytic fungi in grasses and woody plants. Minnesota: APS Press; 1996; 31-65.

24. Leuchmann A, Clay K. *Atkinsonella hypoxylon* and *Balansia cyperi*, epiphytic members of the Balansiae. Mycologia 1988; 80: 192-99.

25. Petrini O. Endophytic fungi in British Ericaceae: a preliminary study. Tran Br Mycol Soc 1984; 83: 510-2.

26. Fisher PJ, Petrini O, Webster J. Aquatic hyphomycetes and other fungi in living aquatic and terrestrial roots of *Alnus glutinosa*. Mycol Res 1991; 95: 543-7.

27. Bertoni MD, Cabral D. Phyllosphere of Eucalyptus viminalis II: Distribution of endophytes. Nova Hedwigia 1988; 46: 491-502.

28. Wildman HG, Parkinson D. Microfungal succession on living leaves *of Populus tremuloides*. Can J Bot 1979; 57: 2800-11.

29. Griffith GS, Boddy L. Fungal decomposition of attached angiosperm twigs I; decay community development in ash, beech and oak. New Phytol 1990; 407-15.

30. Shigo AL. Fungi isolated from oak wilt trees and their effects on *Ceratocystis fagacearum.* Mycologia 1958; 50: 757-69.

31. Cotter HVT, Blanchard RO. The fungal flora of bark of *Fagus grandiflora* Mycologia 1982; 74: 836-43.

32. Parkinson D, Kendrick WB. Investigation of soil microhabitats. In: Parkinson D, Waid JS, editors. The ecology of soil fungi. Liverpool: Liverpool Uni Press; 1960: 22-8.

33. Kendrick WB, Burgess A. Biological aspects of the decay of *Pinus sylvestris* leaft litter. Nova Hedwigia 1962; 4: 313-42.

34. Tsao PH. Selective media for isolation of pathogenic fungi. Annu Rev Phytopathol 1970; 8: 157-86.

35. Tuite J. Plant pathological methods. Minneapolis; Burgess Publishing Co: 1968.

36. Anonymous. Food mycology. A guide to the use of Oxoid culture media. Basingstoke: Unipath Limited; 1990.

37. Filtenborg O, Frisvad JC, Thrane U. The significant of yeast extract composition on metabolite production in Penicillium and Aspergillus classification. New York: Plenum Publishing; 1990; 433-41.

38. Scott PM, Lawrence JW, van Walbeck W. Detection of mycotoxins by thinlayer chromatography: application to screening of fungal extracts. Appli Microbiol 1970; 220: 280-3.

39. Smith D, Onions ASH. The preservation and maintenance of living fungi. Kew: Commonweath mycological institute; 1983.

40. Landecker EM. Fundamentals of the fungi 4th. New Jersey: Prentice-Hall Inc; 1996.

41. Paterson RRM, Bridge PD. Biochemical techniques for filamentous fungi. Wallingford: CAB International; 1994.

42. D'Mello JPF, Macdonald AMC. Mycotoxin. Animal Feed Sci Technol 1997; 69: 155-66.

43. Vandamme EJ. The search for novel microbial fine chemicals, agrochemicals and biopharmaceuticals. J Biotechnol 1994; 37: 89-108.

44. Dreyfuss MM, Chapela IH. Potential of fungi in the discovery of novel, low molecular weight pharmaceuticals. In: Gullo VP, editor. The discovery of natural products with therapeutic potential. Boston, MA: Butterworth-Heinemann; 1994; 49-80.

45. Stierle A, Strobel G. The search for a taxol- producing microorganism among the endophytic fungi of the Pacific yew, *Taxus brevifolia*. J Nat Prod 1995 Sep; 58 (9): 1315-24.

46. Kingston DGI. Taxol: the chemistry and structure-actively relationships of a novel anticancer agent. Trends Biote 1994 Jun; 12: 222-7.

47. Costello M, Kellmel K. Medical attributes of *Taxus brevifolia*, the Pacific yew [Online]. Wilkes-Barre, PA; 1997 Jul. Available from: http://wilkes1.edu/~kklemow/Taxus.html [Accessed 1998 Jan 1].

48. Cragg GM, Schepartz SA, Suffness M, Grever MR. The taxol supply crisis. New NCI pplicies for handling the large- scale production of novel natural product anticancer and anti- HIV agents. J Nat Prod 1993 Oct; 56 (10): 1657-68.

49. Panda D, Miller HP, Islam K, Wilson L. Stabilization of microtubute dynamics by estramustine by binding to a novel sites in tubulin: A possible mechanistic basis for its antitumor action. Proc Natl Acad Sci USA 1997 Sep; 94: 10560-4.

50. Azu M, Essess J. Taxol [Online]. University of Missouri Columbia; 1997 May. Available from: http://www.missouri.edu/~chemrg/210W97/taxol_bodypage.htm#synthesis [Accessed 1998 Jan 3].

51. Strobel GA, Hess WM, Ford E, Sidhu RS, Yang X. Taxol from fungal endopyhtes and the issue of biodiversity. J Indus Microbiol 1996; 17: 417-23.

52. Li JY, Strobel G, Sidhu R, Hess WM, Ford EJ. Endophytic taxol- producing fungi from bald cypress, *Taxodium distichum*. Microbiology 1996; 142: 2223-6.

53. Ball OJP, Prestidge RA, Sprosen JM. Interrelationships *between Acremonium lolii*, Peramine, and Lolitrem B in Perennial Ryegrass. Appl Environ Microbiol 1995 Apr; 61 (4): 1527-33.

54. Porter JK. Chemical constituents of grass endophytes. In: Bacon CW, White Jr JF, editors. Biotechnology of endophytic fungi of grasses. Florida: CRC Press Inc; 1994; 103-23.

55. Miles CO, di Menna ME, Jacobs SWL, Garthwaite I, Lane GA, Prestidge RA, et al. Endophytic fungi in indigenous Australian grasses associated with toxicity to livestock. Appl Environ Microbiol 1998 Feb; 64 (2): 601-6.

56. Krohn K, Florke U, John M, Root N, Steingrover K, Aust HJ, et al. Biologically active metabolites from fungi: Part 16: New preussomerins J, K and L from an endophytic fungus: structure elucidation, crystal structure analysis and determination of absolute configuration by CD calculations. Tetrahedron 2001; 57: 4343-8.

57. Li Jy, Strobel G, Harper J, Lobkovsky E, Clardy J. Cryptocin, a potent tetramic acid antimycotic from the endophytic fungus *Crytosporiopsis* cf. *quercina*. Org Let 2000; 2 (6): 767-70.

58. Johnson JA, Whitney NJ. Cytotoxicity and insecticidal activity of endophytic fungi from black spruce (*Picca mariana*) needles. Can J Microbiol 1994; 40: 24-7.

59. Varma A, Sudha S, Sahay N, Butehorn B, Franken P. *Piriformospora indica*, a cultivable plant- growth- promoting root endophyte. Appl Environ Microbiol 1999 Jun; 65 (6): 2741-4.

60. Christensen CM. Studies on the biology of *Valsa sordida* and *Cytospora chrysosperma*. Phytopathology 1940; 30: 459-75.

61. Chapela IH, Boddy L. Fungal colonization of attached beech branches. I. Early stages of development of fungal communities. New Phytol 1988; 110: 39-45.

62. Anderson DL, French DW. Isolation of *Hypoxylon mammatum* from aspen stem sections. Can J Bot 1972; 50: 1971-2.

63. Chapela IH. Fungal in healthy stems and branches of American beech and aspen: a comparative study. New Phytol 1989; 113: 65-73.

64. Chapela IH, Boddy L. Fungal colonization of attached beech branches. II. Spatial and temporal organization of communities arising from latent invades in bark and functional sapwood under different moisture regimes. New Phytol 1988; 110: 47-57.

65. Cerkausksas RF, Sinclair JB. Use of paraqual to aid detection of fungi in soybean tissue. Phytopathology 1980; 70: 1036-8.

66. Barnett HL. Hypoxylon punctulatum and its conidial stage on dead oak trees and in culture. Mycologia 1957; 49: 588-95.

67. Petrini O. Notes on some species of Chloroscypha endophytic in Cupressaceae of Europe and North America. Sydowia 1982; 35: 206-22.

68. Sherwood- Pike M, Stone JK, Carroll GC. *Rhabdocline parkeri*, a ubiquitous foliar endophyte of Douglas-fer. Can J Bot 1986; 64: 1849-55.

69. Sridhar KR, Barlocher F. Endophytic aquatic hyphomycetes of roots of spruce, birch and maple. Mycol Res 1992; 96: 305-8.

70. Uecker FA. A limed sequence of development of *Diaporthe phaseolorum* (Diaporthaceae) from *Stokesia laevis*. Mem New York Bot Gard 1989; 49: 38-50.

71. Wilson AD, Clement SL, Kaiser WJ. Survey and detection of endophytic fungi in *Lolium germplasm* by direct staining and aphid assays. Plant Dis 1991; 75: 169-73.

72. Latch GCM, Potter LR, Taylor BF. Incidence of endophytes in seeds from collections of *Lolium* and *Festuca* species. Ann Appl Biol 1985; 111: 59-64.

73. Carroll GC. Fungla associates of woody plants as insect antagonists in leaves and stem. In: Barbosa P, Krischik VA, Jones CG, editors. Microbial mediation of plant- herbivore interactions. New York; John Wiley& Sons Inc: 1991; 253-71.

74. Barnett HI. Isolation and identification of the oak wilt fungus. West Virginia Agricultural Experiment Station Bulletin 1953; 359 T: 1-15.

75. Golengerg EMD, Giannasi MT, Clegg CJ, Smiley M, Durbin M, Henderson D, et al. Chloroplast DNA sequence from a miocene *Magnolia* species. Nature 1990; 344: 656-8.

76. Hendriks L, Goris A, van de Peer Y, Neefs JM, Vancanneyt M, Kerrters K, et al. Phylogenetic relationships among ascomycetes and ascomycete- like yeasts as deduced from small ribosomal subunit RNA sequences. Syst Appl Microbiol 1992; 15: 98-104.

77. Sandhu GS, Kline BC, Stockman L, Roberts GD. Molecular probes for diagnosis of fungi infections. J Clin Microbiol 1995; 33: 2913-9.

78. Vilgalys R, Hester M. Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several Cryptococcus species. J Bacteriol 1990; 172: 4238-46.

79. White T, Bruns T, Lee S, Taylor J. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis M, Gelfand D, Sninshy J, White T, editors. PCR protocols. New York; Academic Press Inc: 1990; 315-22.

80. van de Peer Y, Chapelle S, de Wachter R. A quantitative map of nucleotide substitution rates in bacterial rRNA. Nucleic Acids Res 1996; 24: 3381-91.

81. van de Peer Y, Jansen J, de Rijk J, de Wachter R. Database on the structure of small ribosomal subunit RNA. Nucleic Acids Res 1997; 25: 111-6.

82. Guthric PAI, Magill CW, Frederiksen RA, Odvody GN. Random amplifified polymorphic DNA markers: a system for identifying and differentiating isolates of *Colletotrichum graminicola*. Phytopathology 1992; 82: 832-5.

83. Assigbetse KB, Fernandez D, Dubois MP, Geiger JP. Differentiation of *Fusarium oxysporum* f. sp. *vasinfectum* races on cotton by random amplified polymorphic DNA (RAPD) analysis. Phytopathology 1994; 84:622-6.

84. Nicholson P, Rezanoor HN. The use of random amplified polymorphic DNA to identify pathotype and detect variation in *Pseudocercosporella herpotrichoides*. Mycological Res 1994; 98: 13-21.

85. Lehman PF, Lin D, Lasker BA. Genotypic identification and characterization of species and strains within the genus *Candida* by using random amplified polymorphic DNA. J Clin Micbobiol 1992; 30: 3249-54.

86. Meyer W, Mitchell TG, Freedman EZ, Vilgays R. Hybridization probes for conventional DNA fingerprinting used as single primers in the polymerase chain reaction to distinguish strains of *Cryptococcus neoformans*. J Clin Microbiol 1993; 31: 2274-80.

87. Majer D, Mithen R, Lewis BG, Vos P, Oliver RP. The use of RFLP fingerprinting for the detection of genetic variation in fungi. Mycol Res 1996; 100: 1107-11.

88. Mueller UG, Lipari SE, Milgroom MG. Amplified fragment length polymorphism (AFLP) fingerprinting of symbiotic fungi cultured by the fungus-growing ant *Cyphomyrmex minutus*. Mol Eco 1996; 5: 119-22.

89. Moon CD, Tapper BA, Scott B. Identification of Epichloe endophytes in planta by a microsatellite-based PCR fingerprinting assay with automated analysis. Appl Environ Microbiol 1999 Mar; 65 (3): 1268-79.

90. Bruns TD, Vilgalys R, Brans SM, Gonzalez D, Hibbert DS, Lane DJ, et al. Evolutionary relationships within fungi: analysis of nuclear small subunit rRNA sequences. Mol Phylo Evol 1992; 1: 231-41.

91. Hibbert DS. Ribosomal RNA and fungus systematics. Transactions of the Mycological Society of Japan 1992; 33: 533-56.

92. Gaudet J, Julien J, Lafay JF, Brygoo Y. Phylogeny of some Fusarium species, as determined by large subunits rRNA sequence comparison. Mol Biol Evol 1989; 6: 227-42.

93. Bowman BH, Taylor JW, Browilee AG, Lee J, Lu S, White TJ. Molecular evolution of the fungi: relationship of basidiomycetes, ascomycetes and chytridiomycetes. Mol Biol Evol 1992; 9: 285-96.

94. Buchko J, Klassen GR. Detection of length heterogeneity in the ribosomal DNA of *Pythium ultimum* by PCR amplification of the intergenic region. Curr Gene 1990; 18: 203-5.

95. Spreadbury CL, Bainbridge BW, Cohen J. Restriction fragment length polymorphisms in isolates of *Aspergillus fumigatus* probed with part of the intergenic spacers region from the ribosomal RNA genes complex of *Aspergillus nidulans*. J Gen Microbiol 1990; 136: 1991-4.

96. Nazar RN, Hu X, Schmidt J, Culham D, Robb J. Potential use of PCR amplified ribosomal intergenic sequences in the detection and differentiation of *Verticillium* wilt pathogen. Physiol Mol Plan Patho 1991; 39: 1-11.

97. Taylor JW, White TJ. Molecular evolution of nuclear and mitochondrial ribosomal DNA region in *Neurospora* and other *Sordariceae* and comparison with the mushroom, *Laccaria*. Fun Gene Newslet 1991; 38: 26.

98. Anderson JB, Stasorski E. Molecular phylogeny of Northern Hemi-sphere species of *Armillaria*. Mycologia 1992; 84: 505-76.

99. Bridge PD, Arora DK. Interpretation of PCR methods for species definition. In: Bridge PD, Arora DK, Reddy CA, Elander RP, editors. Application of PCR in mycology. Wallingford UK: CAB International Pub; 1998; 63-84.

100. Mitchell JI, Roberts PJ, Moss ST. Sequence or structure? A short review on the application of nucleic acid sequence information to fungal taxonomy. Mycologist 1995 May; 9 (Part 2).

101. Garden M, Bruns TD. ITS primers with enhanced specificity for basiomycetes application to the identification of mycorrhizae and rusts. Mol Eco 1993; 2: 113-8.

102. Gardes M, Bruns TD. Rapid characterization of ectomycorrhizae using RFLP pattern of their PCR amplified-ITS. Mycol Socie Newslet 1991; 41: 14.

103. Gardes M, White TJ, Fortin JA, Bruns TD, Taylor JW. Identification of indigenous and introduced symbiotic fungi in ectomycorrhizae by amplification of nuclear and mitochondrial ribosomal DNA. Can J Bot 1991; 69: 180-90.

104. Lee SB, Taylor JW. Phylogeny of five fungus-like protoctistan *Phytophthora* species, inferred from the internal transcribed spacers of ribosomal DNA. Mol Bol Evo 1992; 9: 636-53.

105. Bruns TD, White TJ, Taylor JW. Fungal molecular systematics Annual Review of Ecology and Systematics 1991; 22: 525-64.

106. Sreenivasaprasad S, Sharada K, Brown AE, Mills PR. PCR- based detection of *Colltotrichum acutatum* on strawberry. Plant Pathology 1996; 45: 650-5.

107. Hamelin RC, Berbe P, Gignae M, Bourassa M. Identification of root rot fungi in nursery seedlings by nested multiplex PCR. Appl Environ Microbiol 1996 Nov; 62 (11): 4026-31.

108. Jackson CJ, Barton RC, Evans EGV. Species identification and differentiation of dermatophyte fungi by analysis of ribosomal-DNA intergenic spacer regions. J Clin Microbiol 1999 Apr; 37 (4): 931-6.

109. Arora DK, Hirsch PR, Kerry BR. PCR-based molecular discrimination of *Verticillium chamydosporium* isolates. Mycol Res 1996; 100: 801-9.

110. Appel DJ, Gordon TR. Intraspecific variation within population of *Fusarium oxysporum* based on RFLP analysis of intergenic spacer region of the rDNA. Experimen Myco 1995; 19: 120-8.

111. Klassen GR, Buchko J. Subrepeat structure of the intergenic region in the ribosomal DNA of the oomycetous fungi *Pythium ultimum*. Curr Gene 1990; 17: 125-7.

112. Bridge PD. An evaluation of some physiological and biochemical methods as an aid to the characterization of species of *Penicillium* subsection Fasciculata J Gen Microbiol 1985; 131: 1887-95.

113. Paterson RRM. Standardization one- and two-dimension thin-layer chromatographic methods for the identification of secondary metabolites in *Penicillium* and other fungi. J Chromato 1986; 368: 1599-609.

114. de Hoog GS, van den Ende AHG Gerrits. Nutritional pattern and eco-physiology of *Hortaea wenechii*, agent of human tinea nigra. Anton Leeuw Int J G 1992; 62: 321-9.

115. de Hoog GS, van den Ende AHG Gerrits, Uijthof JMJ, Untereiner WA. Nutritional physiology of the type isolates of currently accepted species of *Exophiala* and *Phaeocococcomyces*. Antonie Leeuwenhoek Int J Genet 1995; 68: 43-9.

116. Yurlowa NA, de Hoog GS. Anew variety of *Aureobasidium pullulans* characterized by exopolysaccharide structure, nutritional physiology and molecular features. Antonic Leeuwenhoek Int J Genet 1997; 72: 141-7.

117. McGinnis MR, Salkin IF. Identification of molds commonly used in proficiency tests. Lab Med 1986; 17: 138-42.

118. Millner PC, Motta JJ, Lentz PL. Ascospores, germ pores, ultrastructure and thermophilism of *Chaetomium*. Mycologia 1977; 69: 720-33.

119. Pitt JI. An appraisal of identification methods for *Penicillium* species: novel taxonomic criteria based on temperature and water relations. Mycologia 1973; 65: 1135-57.

120. Espinel-Ingroff A, McGinnis MR, PincusDH, Goldson PR, Kerkering TM. Evaluation of the API 20C yeast identification system for the differentiation of some damatiaceous fungi. J Clin Microbiol 1989; 27: 2565-9.

121. Rath AC, Carr CJ, Graham BR. Characterization of *Metarrhizium anisopliae* strains by carbohydrate utilization (API 50 CH). J Invertebr Pathol 1995; 65: 152-61.

122. Bridge PD, Hawksworth DL. New horizons in biosystematics of filamentous fungi. Biopapers 1990; 10: 9-12.

123. Frisvad JC. Classification of organism by secondary metabolites. In: Hawksworth DL, editor. The identification and characterization of pest organisms. Wallingford UK: CAB International Pub; 1994; 303-21.

124. Whalley AJS, Edwards RL. Secondary metabolites and systematic arrangement within the *Xylariaceae*. Can J Bot 1995; 73 (Suppl. 1): S802-10.

125. Mugnai L, Bridge PD, Evans HC. A chemosystematic evalution of the genus *Beauvria*. Mycol Res 1989; 92: 199-209.

126. Lechevalier H, Lechevalier MP. Chemotaxonomic use of lipids-an overview. In: Ratledge C, Wilkinson SG, editors. Microbial lipids vol 1. London UK: Academic Press Ltd; 1988; 869-902.

127. Amano N, Shinmen Y, Akimoto K, Kawashima H, Amachi T. Chemotaxonomic significance of fatty acid composition in the genus *Mortierella* (Zygomycetes, Mortierellaceae). Mycotaxon 1992; 44: 257-65.

128. Augustyn OPH, Kock JFL, Ferreira D. Differentiation between yeasts species and strains within a species by cellular fatty acid analysis 3. *Saccharomyces sensu lato*, *Arxiozyma* and *Pachytichospora*. Syst Appl Microbiol 1990; 13: 44-55.

129. Blomquist G, Andersson B, Andersson K, Brondz I. Analysis of fatty acids. A new method for characterization of moulds. J Microbiol Methods 1992; 16: 59-68.

130. Stahl PD, Klug MJ. Characterization and differentiation of filamentous fungi based on fatty acid composition. Appl Environ Microbiol 1996; 62: 4136-46.

131. Carlile MJ, Watkinson SC. The fungi. London UK: Academic Press Ltd; 1994.

132. Kohn LM. Developing new characters for fungal systematics: an experimental approach for determining the rack of resolution. Mycologia 1992; 84: 139-53.

133. Strobel GA, Torczynski, Bollon. *Acremonium* sp. leucinostatin A producing endophyte of European yew (*Taxus baccata*). Plant Sci 1997; 128: 97-108.

134. Helander ML, Sieber TN, Petrini O, Neuvonen S. Endophytic fungi in Scots pine needles: spatial variation and consequence of simulated acid rain. Can J Bot 1994; 72: 1108-13.

135. Smith D, Onions ASH. The preservation and maintains if living fungi 2ed. Wallingford UK: CAB International; 1994.

136. Wood GL, Washington JA. Antibacterial susceptibility tests: Dilution and disk diffusion methods. In: Murray PR, Pfaller MA, Tenover FC, Yolken RH, editors. Manual of clinical microbiology 6 ed. Washington DC: ASM Press; 1995; 1327-41.

137. Espninel-Ingroff A, Pfaller MA. Antifungal agents and susceptibility testing. In: Murray PR, Pfaller MA, Tenover FC, Yolken RH, editors. Manual of clinical microbiology 6 ed. Washington DC: ASM Press; 1405-14.

138. Collins LA, Franzblau SG. Microplate Alamar Blue assay versus BACTEC 460 system for hight-throughput screening of compounds against *Mycobacterium tuberculosis* and *Mycobacterium avium*. Antimicrob Agents Chemother 1997 May; 41 (5): Inpress.

139. Serotec product datasheet. Alamar Blue™. MD US: Biowhittaker Inc; 1999.

140. Trager W, Jensen JB. Human malarial parasites in continuous culture. Science 1976: 193: 673-5.

141. Lambros C, Vavderberg JP. Synchronization of *Plasmodium falciparum* erythrocytic stage in culture. J Parasitol 1979; 65 (3): 418-20.

142. Rowe E E, Kellner A. Liquid nitrogen preservative of red blood cells for transfusion; a low glycerol rapid freeze procedure. Crybiology 1968; 5: 119-28.

143. Connell MPE, Fabiano E. Antimalarial activity in crude extracts of Malawian medicinal plants. Ann Trop Med Parasitol 1969; 90 (66): 597-602.

144. Chen M, Theander TG, Christensen SB, Hviid L, Kharazmi A, Licochalone A. A new antimalarial agent, inhibits *in vitro* growth of the human malaria parasite *Plasmodium falciparum* and protcets mice from *P. yoelii* infection. Anticrob agents Chemothe 1994: 38 (7): 1470-5.

145. Chilils GE, Hausler B, Milhous W, Chen C, Wimonwattrawatee T, Pooyindee N, et al. In vitro activity of pyronaridine against field isolates and reference clones of Plasmodium falciparum Am J Trop Hyg 1988; 38 (1): 24-9.

146. Schlichtherle M, Wahlgren M, Perlmann H, Scherf A. Method in malaria research 3ed. University Boulevard, Manassas, VA: American Type Culture Collection; 2000.

147. Lee SB, Taylor JW. Isolation of DNA from fungal mycelia and single spore. In: Innis MA, Gelfand DH, Sinnsky JJ, White TJ. PCR protocols; a guide to methods and applications. California: Academic Press Inc; 1990; 282-7.

148. Meinkoth J, Wahl G. Hybridization of nucleic acids immobilized on solid supports. Anal Biochem 1988; 138: 267-84.

149. Thomson JD, Higgins DG, Gibson TJ. Clastal W: Improving the senitivity of progressive, positions-specific gap penalties and weight matrix choice. Nuclic Acids Res. 1994; 22: 4673-80.

150. Felsenstein, J. PHYLIP-Phylogeny Inference Package (Version 3.2). Cladistics 5: 164-166 1989.

151. Rieckmann KH. Determination of the drug sensitivity of *Plasmodium falciparum.* JAMA 1971; 217: 573-8.

152. O' Neill MJ, Bray DH, Boardman P, Philipson JD, Warhurst DC. Plants as sources of antimalarial drugs part I. In vitro test method for the evaluation of crude extract from plants. Planta Med 1985; 5: 394-9.

153. Mitchell JI, Roberts PJ, Moss ST. Sequence or structure? A short review on the application of nucleic acid sequence information to fungi taxonomy. Mycologist 1995 May; 9: Part 2.

154. ejuarezc@inta.gov.ar, *Diaporthe phaseolorum* var. *meridionalis.* [Online] University of Illinois; 1997 Mar. Available from: http://www.ag.uiuc.edu/archives/experts/crop/0025.html [Accessed 2001 Oct 10].

155. Gulya TJ, Masirevic S. Disease of sunflower (*Helianthus annuus* L.) and Jerusalem Artichoke (*H. tuberosus* L.). [Online]. The American Phytopathological Society; 1993 Mar. Available from: http://www.scisoc.org/resource/common/names/sunflower.htm [Accessed 2001 Oct 23].

156. Preisig O, Moleleki N, Smit WA, Wingfield BD, Wingfield MJ. A novel RNA mycovirus in a hypovirulent isolate of the plant pathogen *Diaporthe ambigua*. J Gen Virology 2000; 81: 3107-14.

157. GWRDC funded. *Phomopsis viticola*, pathogenicity and management [Online]. SARDI Horticulture Pathology & Nematology; 1999 Sep. Available from: http://www.sardi.sa.gov.au/hort/patholog/proj_pho.htm [Accessed 2001 Oct 23].

158. Jones AL, Sutton TB. Fusicoccum canker, *Phomopsis amygdali* [Online]. Kearneysville tree fruit research and education center, West verginia Uni; Available from: http://www.caf.wvu.edu/kearneysville/disease_descriptions/fusicom.html [Access 2001 Oct 23].

159. Mostert L, Crous P. *Phomopsis* (dead arm): new facets of this well-known vine desease [Online]. Wynboer WindLand, South Africa; 2000. Available from: http://www.wynboer.co.za/recentarticles/1000phomopsis.php3 [Accessed 2001 Oct 23]

160. Dorrance AE, Lipps PE, Mills DR. *Phomopsis* seed rot of soybean [Online]. Ohio State Uni. Available from: http://www.ohioline.osu.edu/ac-face/0036.html [Accessed 2001 Oct 23].

161. McManus P. *Monilinia oxycocci* and *Phomopsis vaccinii* [Online] University of Wisconsin-Madison. Available from: http://www.plantpath.wisc.edu/fpath/moniphom.htm [Access 2001 Oct 23].

APPENDIX

Appendix 1

1. Media

1.1 Yeast Extract Sucrose Medium.

Yeast extract	20.0 g
Sucrose	150 g
Distilled water up to	1 L.

1.2 Malt Czapek Medium

Czapek stock solution A	50 ml
Czapek stock solution B	50 ml
Sucrose	30 g
Malt Extract	40 g

Distilled water up to 1 L, adjusted pH to 5.0

Czapek stock solution A

$NaNO_3$	4.0 g
KCl	1.0 g
$MgSO_4\ 7H_2O$	1.0 g
$FeSO_4\ 7H_2O$	0.02 g

Dissolved in distillation water up to	100 ml

Keep in a refrigerator.

Czapek stock solution B

K_2HPO_4	2.0 g
A solution	1.0 ml
B solution	1.0 ml
Dissolved in distillation water up to	100 ml

Keep in a refrigerator.

A solution

$ZnSO_4\ 7H_2O$	1.0 g
Dissolved in distillation water up to	100 ml

B solution

$CuSO_4\ 5H_2O$	0.5 g
Dissolved in distillation water up to	100 ml

1.3 Cornmeal Malt Extract Agar (CMMA)

Cornmeal Agar (Difco)	17.0 g
Malt extract	20.0 g
Yeast extract	2.0 g
Dissolved in distillation water up to	1 L.

1.4 Complete Middlebrook 7H9 broth (7H9GC-tween)

Middlebrook 7H9 broth

Glycerol	0.2 % v/v
Casitone	1.0 g
Tween-80	0.05 % (v/v)
OADC (oleic acid, albumin, daxtroes, catalase)	10 % v/v

Dissolved in distillation water, then autoclaved without OADC.

1.5 Complete RPMI 1640 medium with serum

RPMI 1640 powder (1000 μg/ml of PABA and Folic acid) with L-glutamine and without sodium bicarbonate

(Cat. No. 31800-022; Gibco BBL)	10.4 g
HEPES buffer	5.94 g
Sodium bicarbonate ($NaHCO_3$)	2.0 g
Getamicin solution (stock solution 25 mg/ml)	1 ml

Dissolved in double distilled water up to 900 ml then sterile by filtration pass through Millipore membrane (0.45 μm pore size)), then store frozen (in 90 ml aliquots). For complete RPMI 1640, add 10 % of human serum. The serum was inactivated at 56 °C, for 60 min, then stored at -20 °C.

2. Phosphate buffer saline (PBS) pH 7.2

Na_2HPO_4 (0.067 M)	72 ml
Na_2HPO_4 (0.067 M)	28 ml
NaCl	8.5 g

Distilled water up to 1 L, sterile by autoclaving.

3. Freezing solution.

Sorbitol	3 % (w/v)
NaCl	0.65 % (w/v)
Glycerol	28% (v/v)

To make 250 ml, mixes 180 ml of 4.2 % sorbitol in 0.9 % NaCl with 70 ml of glycerol. Filter-sterilize; store frozen.

4. Field's Stain

Field's solution A

Methylene blue	1.6 g
Azure I, B (Sigma)	1.0 g
Na_2HPO_4 anhydrous	10.0 g
KH_2PO_4 anhydrous	12.5 g
In distillation water up to	1000 ml

Field's solution B

Eosin	2.0 g
Na_2HPO_4 anhydrous	10.0 g
KH_2PO_4 anhydrous	12.5 g
In distillation water up to	1000 ml

5. Method for staining blood smears

1. Fix the thin blood smears in absolute methanol for 1 min.
2. Air dry thin blood smear after fixing.
3. Drip in Field's solution A for 1 min, then rinse with tap water.
4. Drip in Field's solution B for 30 sec, then rinse with tap water.
5. Dry at room temperature.

6. Reagent and buffer for DNA amplification by PCR.

6.1 Lysis buffer

Tris-HCl (pH 7.2)	50 mM
EDTA	50 mM
SDS	3 %
2-mercaptoethanol	1 %

6.2 Chloroform : TE-saturated phenol 1:1, v/v

6.3 TE for resuspending pellet

Tris-HCl	10 mM
EDTA	0.1 mM

6.4 Gel loading buffer

Bromophenol blue	0.25 %
Sucrose in water	40% (w/v)

Store temperature at 4°C

6.5 5X Tris-Borate Buffer (TBE)

Tris base	54 g
Boric acid	27.5 g
0.5 M EDTA pH 8.0	20 ml

The working solution was 1X TBE, dilute with four volume of distilled water.

6.6 10X Buffer

Tris HCl pH 9.0	100 mM
KCl	500 mM
Triton X-100	1%

6.7 2 mM dNTP (dATP, dCTP, dGTP, dTTP mix)

dATP	100 mM
dCTP	100 mM
dGTP	100 mM
dTTP	100 mM

Mixed equal volume of each dNTP to get 25 mM dNTP, then dilute to 2 mM dNTP with sterile double distilled water.

Appendix II

Table 14. Raw data for antibacterial and antifungal activity from culture broth extract of MCz medium

No.	Isolate	Media	Test microorganisms						
			SA	EF	BS	EC	PA	CA	TM
1	Acam 02	MCz	I	W	S	I	I	I	W
2	Acam 03	MCz	S	S	S	I	I	I	S
3	Acam 04	MCz	I	I	S	I	I	I	W
4	Acam 06	MCz	S	S	S	I	I	S	S
5	Acam 08	MCz	I	I	S	I	I	I	S
6	Acam 09	MCz	I	I	I	I	I	I	S
7	Acam 11	MCz	M	S	S	I	I	I	S
8	Achi 01	MCz	I	I	S	I	I	I	M
9	Achi 02	MCz	I	I	S	I	I	I	S
10	Afla 01	MCz	S	S	S	S	S	I	S
11	Alan 01	MCz	S	S	S	I	I	S	S
12	Alan 02	MCz	I	I	I	M	I	I	W
13	Alan 06	MCz	I	I	S	I	I	I	S
14	Alan 07	MCz	I	M	S	I	I	I	S
15	Amac 02	MCz	I	I	I	I	I	S	I
16	Amac 04	MCz	S	S	S	I	I	M	S
17	Amac 05	MCz	I	I	W	I	I	M	W
18	Amac 09	MCz	I	I	S	I	I	I	I
19	Atag 01	MCz	M	I	S	I	I	I	I
20	Ccre 01	MCz	S	S	S	I	I	S	S
21	Ccre 02	MCz	S	M	S	I	I	S	S
22	Ccre 03	MCz	S	M	S	I	I	S	S
23	Ccre 04	MCz	I	S	I	I	I	I	I
24	Ccre 05	MCz	W	S	S	I	I	I	S
25	Ccre 06	MCz	W	S	S	I	I	I	M
26	Ccre 09	MCz	S	I	S	I	I	I	I
27	Cinc 03	MCz	S	I	W	I	I	S	S
28	Cinc 05	MCz	I	I	S	I	I	I	M
29	Cpan 03	MCz	I	M	S	I	I	I	S
30	Cpan 04	MCz	I	I	S	I	I	I	W
31	Cpan 05	MCz	I	I	S	I	I	I	I
32	Cpan 06	MCz	S	I	S	I	I	I	I
33	Cpan 08	MCz	I	I	S	I	I	I	I
34	Cpan 09	MCz	W	I	S	I	I	I	I
35	Dfil 03	MCz	S	I	S	I	I	W	S

No.	Isolate	Media	Test microorganisms						
			SA	EF	BS	EC	PA	CA	TM
36	Dfil 05	MCz	S	I	S	I	I	I	I
37	Dfil 08	MCz	S	S	S	I	I	I	W
38	Dfil 09	MCz	S	S	S	I	I	I	S
39	Elon 01	MCz	S	S	M	I	I	I	S
40	Elon 03	MCz	M	M	S	I	I	S	S
41	Esca 02	MCz	S	S	S	I	I	S	S
42	Esca 03	MCz	S	I	S	S	I	S	M
43	Esca 05	MCz	I	I	I	I	I	I	S
44	Haro 03	MCz	S	S	S	I	I	S	S
45	Haro 05	MCz	S	S	S	I	I	I	I
46	Ijav 02	MCz	I	I	S	I	I	I	I
47	Ijav 04	MCz	I	W	I	I	I	I	S
48	Ijav 07	MCz	I	S	I	I	I	I	I
49	Mcor 01	MCz	-	-	-	-	-	-	-
50	Mcor 03	MCz	I	I	W	I	I	I	I
51	Mcor 04	MCz	I	I	S	I	I	I	I
52	Mcor 05	MCz	S	S	S	I	I	I	S
53	Mcor 06	MCz	S	S	S	S	S	S	S
54	Mcor 07	MCz	I	I	S	I	I	I	I
55	Mleu 01	MCz	I	I	S	I	I	I	M
56	Mleu 04	-	-	-	-	-	-	-	-
57	Mleu 05	MCz	S	S	S	I	I	I	S
58	Mleu 06	MCz	S	S	S	I	I	M	S
59	Mmal 01	MCz	I	I	W	I	I	I	I
60	Mmal 03	MCz	I	S	S	I	I	I	M
61	Mphi 02	MCz	S	M	S	S	I	S	S
62	Mphi 05	MCz	I	I	S	I	I	S	S
63	Mphi 06	MCz	S	W	S	I	I	S	S
64	Mphi 09	MCz	I	I	S	I	I	S	S
65	Pind 04	MCz	S	S	S	I	I	S	S
66	Pind 05	MCz	S	S	S	S	S	S	S
67	Pind 06	MCz	S	S	S	S	S	S	S
68	Pind 07	MCz	W	S	W	I	I	I	S
69	Sgri 01	MCz	S	S	S	I	I	S	S
70	Sgri 03	MCz	S	S	S	S	M	S	M
71	Sgri 06	MCz	S	W	S	I	I	I	M
72	Sgri 07	MCz	S	M	S	I	I	I	S
73	Sgri 08	MCz	I	I	I	I	I	I	I
74	Sher 01	MCz	I	I	S	I	I	I	I
75	Sher 03	-	-	-	-	-	-	-	-
76	Sher 04	MCz	S	M	S	I	I	S	S
77	Sher 05	MCz	I	I	S	I	I	I	S
78	Sher 06	MCz	I	S	S	I	I	I	I
79	Sill 07	MCz	M	M	M	I	I	I	S
80	Sind 01	MCz	I	I	S	I	I	I	I
81	Sind 02	MCz	S	I	S	I	I	I	S

No.	Isolate	Media	Test microorganisms						
			SA	EF	BS	EC	PA	CA	TM
82	Sind 04	MCz	I	I	I	I	I	I	S
83	Sind 06	MCz	I	I	I	I	I	I	I
84	Sind 07	MCz	I	I	I	I	I	I	I
85	Sinv 01	MCz	I	I	S	I	I	I	I
86	Sinv 02	MCz	I	I	S	I	I	I	W
87	Sinv 03	MCz	M	S	S	I	I	M	S
88	Sluz 01	MCz	I	I	S	I	I	I	S
89	Sluz 02	MCz	S	S	S	I	I	I	S
90	Sluz 03	MCz	I	S	S	I	I	I	S
91	Sluz 05	MCz	S	I	S	I	I	I	S
92	Spin 05	MCz	M	W	S	I	I	I	M
93	Tcam 12	MCz	M	I	S	I	I	I	I
94	Tcri 02	MCz	W	I	S	W	I	I	S
95	Tcri 04	MCz	I	I	I	I	I	W	I
96	Tcri 06	MCz	S	I	S	I	I	S	S
94	Tcri 02	MCz	W	I	S	W	I	I	S
95	Tcri 04	MCz	I	I	I	I	I	W	I
96	Tcri 06	MCz	S	I	S	I	I	S	S
97	Tcri 07	MCz	S	M	S	I	I	S	S
98	Tcri 08	MCz	S	S	S	S	I	S	S
99	Tint 01	MCz	I	I	M	I	I	I	I
100	Tint 02	MCz	M	S	S	I	I	S	S
101	Tint 04	MCz	I	I	S	I	I	I	M
102	Tpal 01	MCz	I	I	I	I	I	I	I
103	Tpal 05	MCz	I	I	I	I	I	I	W
104	Ulob 01	MCz	I	I	S	I	I	I	I
104	Ulob 01	MCz	I	I	S	I	I	I	I
105	Ulob 04	MCz	I	I	S	I	I	I	I
106	Uruf 05	MCz	S	W	S	I	I	I	S
107	Uruf 06	MCz	I	I	I	I	I	I	W
108	Uruf 08	MCz	I	W	I	I	I	I	W
109	Vden 03	MCz	S	S	S	I	I	M	S
110	Vden 04	MCz	I	I	S	I	I	I	W
111	Vden 07	MCz	S	S	S	I	I	M	S
112	Vden 08	MCz	I	M	S	I	I	I	S
113	Vden 10	MCz	M	I	S	I	I	I	W
114	Vden 11	MCz	S	S	S	I	I	I	S
115	Vped 03	MCz	I	S	S	I	I	I	S
116	Vped 05	MCz	I	I	S	I	I	I	I
117	Vped 06	MCz	I	I	S	I	I	I	S
118	Vped 08	MCz	S	S	S	W	I	I	S
119	Wtom 01	MCz	I	I	S	I	I	I	S
120	Wtom 02	MCz	I	I	S	I	I	I	I
121	Wtom 03	MCz	I	I	I	I	I	I	I
122	Wtom 04	MCz	S	S	S	I	I	I	W
123	Wtom 05	MCz	I	I	I	I	I	I	I

Table 15. Raw data for antibacterial and antifungal activity from culture broth extract of YES medium

No.	Isolate	Media	Test microorganisms						
			SA	EF	BS	EC	PA	CA	TM
1	Acam 02	YES	I	W	S	I	I	I	S
2	Acam 03	YES	I	S	I	I	I	I	S
3	Acam 04	YES	S	S	S	I	I	W	S
4	Acam 06	YES	I	M	S	I	I	W	S
5	Acam 08	YES	I	S	S	I	I	I	S
6	Acam 09	YES	I	W	S	I	I	I	S
7	Acam 11	YES	I	S	S	I	I	W	S
8	Achi 01	YES	I	S	S	I	I	I	S
9	Achi 02	YES	I	I	S	I	I	I	S
10	Afla 01	YES	I	I	S	I	I	I	W
11	Alan 01	YES	S	I	S	I	I	M	S
12	Alan 02	YES	I	I	I	I	I	I	W
13	Alan 06	YES	I	I	W	I	I	I	I
14	Alan 07	YES	I	I	S	I	I	I	S
15	Amac 02	YES	I	I	M	I	I	I	S
16	Amac 04	YES	S	S	S	I	I	S	W
17	Amac 05	YES	S	I	S	I	I	W	S
18	Amac 09	YES	I	I	S	I	I	I	S
19	Atag 01	YES	I	I	S	I	I	I	S
20	Ccre 01	YES	I	M	S	I	I	I	M
21	Ccre 02	YES	I	S	I	I	I	I	S
22	Ccre 03	YES	S	S	S	M	I	I	S
23	Ccre 04	YES	S	I	S	I	I	I	S
24	Ccre 05	YES	I	I	S	I	I	M	S
25	Ccre 06	YES	I	I	S	I	I	I	S
26	Ccre 09	YES	S	S	S	S	I	S	S
27	Cinc 03	YES	I	S	S	I	I	I	I
28	Cinc 05	YES	I	I	I	I	I	I	M
29	Cpan 03	YES	W	S	S	S	I	W	S
30	Cpan 04	YES	I	S	S	I	I	I	W
31	Cpan 05	YES	I	I	S	I	I	I	M
32	Cpan 06	YES	S	S	S	S	I	S	S
33	Cpan 08	YES	I	S	S	I	I	I	S
34	Cpan 09	YES	I	I	S	I	I	I	S
35	Dfil 03	YES	S	W	S	I	I	I	I
36	Dfil 05	YES	I	I	S	I	I	I	M
37	Dfil 08	YES	S	S	S	I	I	I	S
38	Dfil 09	YES	S	S	S	I	I	I	S
39	Elon 01	YES	I	S	S	I	I	I	S
40	Elon 03	YES	S	S	S	I	I	S	M
41	Esca 02	YES	I	S	S	I	I	S	S

No.	Isolate	Media	Test microorganisms						
			SA	EF	BS	EC	PA	CA	TM
42	Esca 03	YES	S	S	S	I	I	I	S
43	Esca 05	YES	I	I	I	I	I	I	I
44	Haro 03	YES	S	S	S	I	I	S	I
45	Haro 05	YES	I	I	S	I	I	I	I
46	Ijav 02	YES	I	I	I	I	I	I	I
47	Ijav 04	YES	S	I	M	W	I	I	S
48	Ijav 07	YES	I	I	I	I	I	I	I
49	Mcor 01	YES	S	S	S	S	W	S	S
50	Mcor 03	-	-	-	-	-	-	-	-
51	Mcor 04	YES	S	S	S	S	I	S	S
52	Mcor 05	YES	S	S	S	I	I	W	S
53	Mcor 06	YES	S	S	S	S	S	S	S
54	Mcor 07	YES	I	I	S	I	I	I	S
55	Mleu 01	YES	I	I	S	I	I	I	S
56	Mleu 04	YES	S	I	S	I	I	W	M
57	Mleu 05	YES	I	I	S	I	I	I	M
58	Mleu 06	YES	S	S	S	I	I	I	S
59	Mmal 01	YES	S	S	S	W	I	S	S
60	Mmal 03	YES	S	I	S	I	I	I	S
61	Mphi 02	YES	S	S	S	I	I	I	I
62	Mphi 05	YES	I	I	M	I	I	S	M
63	Mphi 06	YES	S	I	S	I	I	S	I
64	Mphi 09	YES	S	S	S	I	I	S	S
65	Pind 04	YES	I	I	S	I	I	I	S
66	Pind 05	YES	S	S	S	S	S	I	S
67	Pind 06	YES	S	S	S	S	S	S	S
68	Pind 07	YES	I	S	I	I	I	M	W
69	Sgri 01	YES	S	M	S	I	I	S	S
70	Sgri 03	YES	I	I	S	I	I	S	S
71	Sgri 06	YES	S	W	S	I	I	I	S
72	Sgri 07	YES	W	I	S	I	I	I	S
73	Sgri 08	YES	I	I	I	I	I	I	I
74	Sher 01	YES	-	-	-	-	-	-	-
75	Sher 03	YES	M	I	S	S	I	W	I
76	Sher 04	YES	S	S	S	I	I	S	S
77	Sher 05	YES	S	S	S	I	I	I	S
78	Sher 06	YES	I	I	S	I	I	I	I
79	Sill 07	YES	W	I	S	I	I	I	S
80	Sind 01	YES	I	I	I	I	I	I	I
81	Sind 02	YES	I	W	I	I	I	I	I

No.	Isolate	Media	Test microorganisms						
			SA	EF	BS	EC	PA	CA	TM
82	Sind 04	YES	I	W	I	I	I	I	I
83	Sind 06	YES	I	I	I	I	I	I	S
84	Sind 07	YES	I	I	I	I	I	I	I
85	Sinv 01	YES	S	I	S	I	I	I	M
86	Sinv 02	YES	I	I	S	I	I	I	S
87	Sinv 03	YES	I	I	S	I	I	W	S
88	Sluz 01	YES	I	I	S	I	I	I	I
89	Sluz 02	YES	S	I	S	I	I	I	S
90	Sluz 03	YES	I	I	S	I	I	I	I
90	Sluz 03	YES	I	I	S	I	I	I	I
91	Sluz 05	YES	I	I	S	I	I	I	S
92	Spin 05	YES	-	-	-	-	-	-	-
93	Tcam 12	YES	I	I	M	I	I	I	S
94	Tcri 02	YES	I	I	S	I	I	I	W
95	Tcri 04	YES	I	I	I	I	I	I	I
96	Tcri 06	YES	S	S	S	I	I	I	S
97	Tcri 07	YES	S	I	S	I	I	S	S
98	Tcri 08	YES	I	I	S	I	I	I	M
99	Tint 01	YES	I	I	S	I	I	I	I
100	Tint 02	YES	I	I	S	I	I	I	W
101	Tint 04	YES	I	I	I	I	I	I	I
102	Tpal 01	YES	I	I	I	I	I	I	I
103	Tpal 05	YES	S	S	S	I	I	I	I
104	Ulob 01	YES	I	S	S	I	I	I	M
105	Ulob 04	YES	I	I	I	I	I	I	W
106	Uruf 05	YES	I	I	S	I	I	I	S
107	Uruf 06	YES	I	I	I	I	I	I	S
108	Uruf 08	YES	I	I	I	I	I	I	S
109	Vden 03	YES	S	S	S	I	I	W	S
110	Vden 04	YES	I	S	S	I	I	I	S
111	Vden 07	YES	W	I	S	I	I	I	I
112	Vden 08	YES	S	S	S	I	I	S	S
113	Vden 10	YES	S	S	S	M	I	M	S
114	Vden 11	YES	I	I	S	I	I	I	S
115	Vped 03	YES	S	S	S	S	I	I	S
116	Vped 05	YES	I	M	S	I	I	I	I
117	Vped 06	YES	I	I	I	I	I	I	S
118	Vped 08	YES	I	I	S	M	I	I	S
119	Wtom 01	YES	I	I	S	I	I	I	M
120	Wtom 02	YES	I	I	I	I	I	I	M
121	Wtom 03	YES	S	S	S	S	S	S	S
122	Wtom 04	YES	I	I	S	I	I	I	M
123	Wtom 05	YES	I	I	I	I	I	I	S
123	Wtom 05	YES	I	I	I	I	I	I	S

Table 16. Raw data for antibacterial and antifungal activity from mycelium extract of MCz medium

No.	Isolate	Media	Test microorganisms						
			SA	EF	BS	EC	PA	CA	TM
1	Acam 02	MCz	I	I	M	I	I	I	I
2	Acam 03	MCz	I	I	M	I	I	I	I
3	Acam 04	MCz	I	I	S	I	I	I	I
4	Acam 06	MCz	I	I	W	I	I	I	I
5	Acam 08	MCz	I	I	S	I	I	I	I
6	Acam 09	MCz	I	I	I	I	I	I	I
7	Acam 11	MCz	I	I	I	I	I	I	I
8	Achi 01	MCz	I	I	S	I	I	I	I
9	Achi 02	MCz	I	I	I	I	I	I	I
10	Afla 01	MCz	I	I	I	I	I	I	W
11	Alan 01	MCz	I	I	S	I	I	I	I
12	Alan 02	MCz	I	I	I	I	I	I	I
13	Alan 06	MCz	I	I	I	I	I	I	I
14	Alan 07	MCz	I	I	S	I	I	I	I
15	Amac 02	MCz	I	I	S	I	I	I	I
16	Amac 04	MCz	I	I	S	I	I	I	S
17	Amac 05	MCz	I	I	I	I	I	I	I
18	Amac 09	MCz	I	I	I	I	I	I	I
19	Atag 01	MCz	I	I	M	I	I	I	I
20	Ccre 01	MCz	S	I	S	I	I	I	I
21	Ccre 02	MCz	I	I	I	I	I	I	I
22	Ccre 03	MCz	I	I	S	I	I	I	I
23	Ccre 04	MCz	I	I	M	I	I	I	I
24	Ccre 05	MCz	S	S	S	I	I	I	I
25	Ccre 06	MCz	I	I	I	I	I	I	I
26	Ccre 09	MCz	I	I	I	I	I	I	I
27	Cinc 03	MCz	I	I	S	I	I	I	I
28	Cinc 05	MCz	I	I	I	I	I	I	I
29	Cpan 03	MCz	I	I	I	I	I	I	I
30	Cpan 04	MCz	I	I	W	I	I	I	I
31	Cpan 05	MCz	I	I	I	I	I	I	I
32	Cpan 06	MCz	I	I	I	I	I	I	I
33	Cpan 08	MCz	I	I	S	I	I	I	I

No.	Isolate	Media	Test microorganisms						
			SA	EF	BS	EC	PA	CA	TM
34	Cpan 09	MCz	I	I	I	I	I	I	I
35	Dfil 03	MCz	S	I	S	I	I	I	I
36	Dfil 05	MCz	I	I	S	I	I	I	I
37	Dfil 08	MCz	S	I	M	I	I	I	I
38	Dfil 09	MCz	I	I	I	I	I	I	I
39	Elon 01	MCz	I	I	I	I	I	I	I
40	Elon 03	MCz	S	I	S	I	I	I	I
41	Esca 02	MCz	S	I	M	I	I	W	I
42	Esca 03	MCz	I	I	M	I	I	I	I
43	Esca 05	MCz	I	I	I	I	I	I	I
44	Haro 03	MCz	S	S	S	I	I	I	I
45	Haro 05	MCz	I	I	M	I	I	I	I
46	Ijav 02	MCz	I	I	I	I	I	I	I
47	Ijav 04	MCz	I	I	I	I	I	I	I
48	Ijav 07	MCz	I	I	I	I	I	I	I
49	Mcor 01	MCz	I	I	I	I	I	I	I
50	Mcor 03	MCz	I	I	M	I	I	I	I
51	Mcor 04	MCz	I	I	S	I	I	I	I
52	Mcor 05	MCz	I	I	M	I	I	I	I
53	Mcor 06	MCz	S	S	S	I	I	I	W
54	Mcor 07	MCz	I	S	M	I	I	I	I
55	Mleu 01	MCz	I	I	S	I	I	I	I
56	Mleu 04	MCz	-	-	-	-	-	-	-
57	Mleu 05	MCz	I	I	S	I	I	I	I
58	Mleu 06	MCz	I	I	I	I	I	I	I
59	Mmal 01	MCz	I	I	S	I	I	I	I
60	Mmal 03	MCz	I	I	W	I	I	I	I
61	Mphi 02	MCz	I	I	M	I	I	I	I
62	Mphi 05	MCz	I	I	M	I	I	I	I
63	Mphi 06	MCz	I	I	M	I	I	I	I
64	Mphi 09	MCz	I	I	S	I	I	I	I
65	Pind 04	MCz	I	I	S	I	I	I	I
66	Pind 05	MCz	S	S	S	I	I	I	I
67	Pind 06	MCz	S	S	S	I	I	I	I
68	Pind 07	MCz	I	I	S	I	I	I	I
69	Sgri 01	MCz	S	S	S	I	I	W	I

No.	Isolate	Media	Test microorganisms						
			SA	EF	BS	EC	PA	CA	TM
70	Sgri 03	MCz	M	S	S	I	I	I	S
71	Sgri 06	MCz	I	I	S	I	I	I	I
72	Sgri 07	MCz	I	I	I	I	I	I	I
73	Sgri 08	MCz	I	I	M	I	I	I	I
74	Sher 01	MCz	-	-	-	-	-	-	-
75	Sher 03	MCz	I	I	S	I	I	I	I
76	Sher 04	MCz	I	I	M	I	I	I	S
77	Sher 05	MCz	I	I	I	I	I	I	I
78	Sher 06	MCz	I	I	I	I	I	I	I
79	Sill 07	MCz	-	-	-	-	-	-	-
80	Sind 01	MCz	I	I	W	I	I	I	I
81	Sind 02	MCz	I	I	I	I	I	I	I
82	Sind 04	MCz	I	I	M	I	I	I	I
83	Sind 06	MCz	I	I	I	I	I	I	I
84	Sind 07	MCz	I	I	I	I	I	I	I
85	Sinv 01	MCz	I	I	I	I	I	I	I
86	Sinv 02	MCz	I	I	I	I	I	I	I
87	Sinv 03	MCz	I	I	W	I	I	I	I
88	Sluz 01	MCz	I	I	I	I	I	I	I
89	Sluz 02	MCz	M	I	S	I	I	I	S
90	Sluz 03	MCz	I	I	I	I	I	I	I
91	Sluz 05	MCz	I	I	S	I	I	I	I
92	Spin 05	MCz	I	I	M	I	I	I	I
93	Tcam 12	MCz	I	I	S	I	I	I	I
94	Tcri 02	MCz	I	I	I	I	I	I	I
95	Tcri 04	MCz	I	I	W	I	I	I	I
96	Tcri 06	MCz	S	S	S	I	I	I	S
97	Tcri 07	MCz	S	I	S	I	I	I	S
98	Tcri 08	MCz	I	I	M	I	I	I	S
99	Tint 01	MCz	I	S	S	I	I	I	I
100	Tint 02	MCz	I	I	S	I	I	I	I
101	Tint 04	MCz	I	I	I	I	I	I	I
102	Tpal 01	MCz	I	I	I	I	I	I	I
103	Tpal 05	MCz	I	I	I	I	I	I	I
104	Ulob 01	MCz	I	I	I	I	I	I	I

No.	Isolate	Media	Test microorganisms						
			SA	EF	BS	EC	PA	CA	TM
105	Ulob 04	MCz	I	I	I	I	I	I	I
106	Uruf 05	MCz	I	I	I	I	I	I	I
107	Uruf 06	MCz	I	I	I	I	I	I	I
108	Uruf 08	MCz	I	I	S	I	I	I	I
109	Vden 03	MCz	I	I	I	I	I	I	S
110	Vden 04	MCz	I	I	S	I	I	I	I
111	Vden 07	MCz	I	I	S	I	I	I	W
112	Vden 08	MCz	I	I	S	I	I	I	I
113	Vden 10	MCz	I	I	S	I	I	I	I
114	Vden 11	MCz	I	I	I	I	I	I	I
115	Vped 03	MCz	I	I	S	I	I	I	I
116	Vped 05	MCz	I	I	S	I	I	I	I
117	Vped 06	MCz	I	I	I	I	I	I	I
118	Vped 08	MCz	S	S	S	I	I	I	S
119	Wtom 01	MCz	I	I	I	I	I	I	I
120	Wtom 02	MCz	I	I	S	I	I	I	I
121	Wtom 03	MCz	I	I	S	I	I	I	I
122	Wtom 04	MCz	I	I	M	I	I	I	I
123	Wtom 05	MCz	I	I	I	I	I	I	I

Table 17. Raw data for antibacterial and antifungal activity from mycelium extract of YES medium.

No.	Isolate	Media	Test microorganisms						
			SA	EF	BS	EC	PA	CA	TM
1	Acam 02	YES	I	I	I	I	I	I	I
2	Acam 03	YES	I	I	S	I	I	I	I
3	Acam 04	YES	I	I	I	I	I	I	I
4	Acam 06	YES	I	I	I	I	I	I	I
5	Acam 08	YES	I	I	I	I	I	I	I
6	Acam 09	YES	I	I	I	I	I	I	I
7	Acam 11	YES	I	I	S	I	I	I	I
8	Achi 01	YES	I	I	I	I	I	I	I
9	Achi 02	YES	I	I	S	I	I	I	I
10	Afla 01	YES	I	I	I	I	I	I	I
11	Alan 01	YES	I	I	I	I	I	I	I

No.	Isolate	Media	Test microorganisms						
			SA	EF	BS	EC	PA	CA	TM
12	Alan 02	YES	I	I	I	I	I	I	I
13	Alan 06	YES	I	I	I	I	I	I	I
14	Alan 07	YES	I	I	S	I	I	I	I
15	Amac 02	YES	I	I	S	I	I	I	I
16	Amac 04	YES	I	I	I	I	I	I	I
17	Amac 05	YES	I	I	S	I	I	I	I
18	Amac 09	YES	I	I	I	I	I	I	I
19	Atag 01	YES	I	I	I	I	I	I	I
20	Ccre 01	YES	I	I	S	I	I	I	I
21	Ccre 02	YES	I	I	S	I	I	I	I
22	Ccre 03	YES	I	I	I	I	I	I	I
23	Ccre 04	YES	I	I	I	I	I	I	I
24	Ccre 05	YES	I	I	I	I	I	I	I
25	Ccre 06	YES	I	I	I	I	I	I	I
26	Ccre 09	YES	I	I	I	I	I	I	I
27	Cinc 03	YES	I	I	S	I	I	I	I
28	Cinc 05	YES	I	I	S	I	I	I	I
29	Cpan 03	YES	I	I	I	I	I	I	I
30	Cpan 04	YES	I	I	I	I	I	I	I
31	Cpan 05	YES	I	I	I	I	I	I	I
32	Cpan 06	YES	I	I	M	I	I	I	I
33	Cpan 08	YES	I	I	S	I	I	I	I
34	Cpan 09	YES	I	I	I	I	I	I	I
35	Dfil 03	YES	I	I	S	I	I	I	I
36	Dfil 05	YES	I	I	I	I	I	I	I
37	Dfil 08	YES	S	S	S	I	I	I	I
38	Dfil 09	YES	I	I	S	I	I	I	I
39	Elon 01	YES	I	I	I	I	I	I	I
40	Elon 03	YES	I	I	I	I	I	I	I
41	Esca 02	YES	I	I	I	I	I	I	I
42	Esca 03	YES	I	I	I	I	I	I	I
43	Esca 05	YES	I	I	I	I	I	I	I
44	Haro 03	YES	I	I	S	I	I	I	I
45	Haro 05	YES	I	I	I	I	I	I	I
46	Ijav 02	YES	I	I	I	I	I	I	I
47	Ijav 04	YES	I	I	I	I	I	I	I
48	Ijav 07	YES	I	I	I	I	I	I	I
49	Mcor 01	YES	I	I	I	I	I	I	I
50	Mcor 03	YES	I	I	S	I	I	I	I
51	Mcor 04	YES	I	I	I	I	I	I	I
52	Mcor 05	YES	I	I	I	I	I	I	I

No.	Isolate	Media	Test microorganisms						
			SA	EF	BS	EC	PA	CA	TM
53	Mcor 06	YES	I	S	M	I	I	I	I
54	Mcor 07	YES	I	I	I	I	I	I	I
55	Mleu 01	YES	I	I	I	I	I	I	I
56	Mleu 04	YES	I	I	I	I	I	I	I
57	Mleu 05	YES	I	I	S	I	I	I	I
58	Mleu 06	YES	S	S	S	I	I	I	I
59	Mmal 01	YES	I	I	S	I	I	I	I
60	Mmal 03	YES	I	I	S	I	I	I	I
61	Mphi 02	YES	I	I	I	I	I	I	I
62	Mphi 05	YES	I	I	I	I	I	I	I
63	Mphi 06	YES	I	I	I	I	I	I	I
64	Mphi 09	YES	I	I	I	I	I	I	I
65	Pind 04	YES	I	I	S	I	I	I	I
66	Pind 05	YES	I	S	W	I	I	I	I
67	Pind 06	YES	I	S	S	I	I	I	W
68	Pind 07	YES	I	I	I	I	I	I	I
69	Sgri 01	YES	I	I	I	I	I	I	I
70	Sgri 03	YES	I	I	S	I	I	I	I
71	Sgri 06	YES	I	I	S	I	I	I	I
72	Sgri 07	YES	I	I	S	I	I	I	I
73	Sgri 08	YES	I	I	I	I	I	I	I
74	Sher 01	YES	-	-	-	-	-	-	-
75	Sher 03	YES	I	I	I	I	I	I	I
76	Sher 04	YES	I	I	I	I	I	I	I
77	Sher 05	YES	I	I	I	I	I	I	I
78	Sher 06	YES	I	I	M	I	I	I	I
79	Sill 07	YES	-	-	-	-	-	-	-
80	Sind 01	YES	I	I	I	I	I	I	I
81	Sind 02	YES	I	I	M	I	I	I	I
82	Sind 04	YES	I	I	I	I	I	I	I
83	Sind 06	YES	I	I	I	I	I	I	I
84	Sind 07	YES	I	I	M	I	I	I	I
85	Sinv 01	YES	I	I	I	I	I	I	I
86	Sinv 02	YES	I	I	S	I	I	I	I
87	Sinv 03	YES	I	I	M	I	I	I	I
88	Sluz 01	YES	I	I	S	I	I	I	I
89	Sluz 02	YES	I	I	I	I	I	I	I
90	Sluz 03	YES	I	I	I	I	I	I	I
91	Sluz 05	YES	I	I	I	I	I	I	I
92	Spin 05	YES	-	-	-	-	-	-	-

No.	Isolate	Media	Test microorganisms						
			SA	EF	BS	EC	PA	CA	TM
93	Tcam 12	YES	I	I	I	I	I	I	I
94	Tcri 02	YES	I	I	W	I	I	I	I
95	Tcri 04	YES	I	I	M	I	I	I	I
96	Tcri 06	YES	I	I	I	I	I	I	I
97	Tcri 07	YES	S	S	S	I	I	I	I
98	Tcri 08	YES	I	I	I	I	I	I	I
99	Tint 01	YES	I	I	S	I	I	I	I
100	Tint 02	YES	I	I	S	I	I	I	I
101	Tint 04	YES	I	I	I	I	I	I	I
102	Tpal 01	YES	I	I	I	I	I	I	I
103	Tpal 05	YES	I	I	I	I	I	I	I
104	Ulob 01	YES	I	I	I	I	I	I	I
105	Ulob 04	YES	I	I	S	I	I	I	I
106	Uruf 05	YES	I	I	I	I	I	I	I
107	Uruf 06	YES	I	I	I	I	I	I	I
108	Uruf 08	YES	I	I	I	I	I	I	I
109	Vden 03	YES	S	I	M	I	I	I	S
110	Vden 04	YES	I	I	S	I	I	I	I
111	Vden 07	YES	I	I	W	I	I	I	I
112	Vden 08	YES	I	I	I	I	I	I	I
113	Vden 10	YES	I	I	S	I	I	I	I
114	Vden 11	YES	I	I	S	I	I	I	I
115	Vped 03	YES	-	-	-	-	-	-	-
116	Vped 05	YES	I	I	I	I	I	I	I
117	Vped 06	YES	I	I	S	I	I	I	I
118	Vped 08	YES	I	I	I	I	I	I	I
119	Wtom 01	YES	I	I	W	I	I	I	I
120	Wtom 02	YES	I	I	I	I	I	I	I
121	Wtom 03	YES	I	I	I	I	I	I	I
122	Wtom 04	YES	I	I	I	I	I	I	I
123	Wtom 05	YES	I	I	I	I	I	I	I

Table 18. Raw data for antimycobacterium activity of culture broth extract from MCz and YES medium.

No.	Isolates	Media	MIC (µg/ml)	Media	MIC (µg/ml)
1	Acam 02	MCz	500	YES	>500
2	Acam 03	MCz	>500	YES	>500
3	Acam 04	MCz	500	YES	>500

No.	Isolates	Media	MIC (µg/ml)	Media	MIC (µg/ml)
4	Acam 06	MCz	500	YES	500
5	Acam 08	MCz	>500	YES	500
6	Acam 09	MCz	500	YES	>500
7	Acam 11	MCz	250	YES	250
8	Achi 01	MCz	I	YES	I
9	Achi 02	MCz	I	YES	500
10	Afla 01	MCz	125	YES	250
11	Alan 01	MCz	500	YES	250
12	Alan 02	MCz	I	YES	500
13	Alan 06	MCz	>500	YES	I
14	Alan 07	MCz	>500	YES	>500
15	Amac 02	MCz	500	YES	250
16	Amac 04	MCz	500	YES	500
17	Amac 05	MCz	>500	YES	500
18	Amac 09	MCz	I	YES	500
19	Atag 01	MCz	>500	YES	500
20	Ccre 01	MCz	500	YES	I
21	Ccre 02	MCz	500	YES	>500
22	Ccre 03	MCz	250	YES	500
23	Ccre 04	MCz	I	YES	I
24	Ccre 05	MCz	I	YES	500
25	Ccre 06	MCz	I	YES	500
26	Ccre 09	MCz	>500	YES	250
27	Cinc 03	MCz	250	YES	500
28	Cinc 05	MCz	I	YES	I
29	Cpan 03	MCz	>500	YES	500
30	Cpan 04	MCz	500	YES	>500
31	Cpan 05	MCz	>500	YES	500
32	Cpan 06	MCz	500	YES	500
33	Cpan 08	MCz	125	YES	31.25
34	Cpan 09	MCz	I	YES	500
35	Dfil 03	MCz	500	YES	>500
36	Dfil 05	MCz	>500	YES	>500
37	Dfil 08	MCz	>500	YES	>500
38	Dfil 09	MCz	500	YES	250
39	Elon 01	MCz	>500	YES	500
40	Elon 03	MCz	500	YES	I
41	Esca 02	MCz	>500	YES	500
42	Esca 03	MCz	250	YES	500
43	Esca 05	MCz	500	YES	>500
44	Haro 03	MCz	250	YES	500

No.	Isolates	Media	MIC (µg/ml)	Media	MIC (µg/ml)
45	Haro 05	MCz	500	YES	I
46	Ijav 02	MCz	I	YES	I
47	Ijav 04	MCz	I	YES	I
48	Ijav 07	MCz	I	YES	I
49	Mcor 01	MCz	-	YES	I
50	Mcor 03	MCz	I	YES	-
51	Mcor 04	MCz	I	YES	250
52	Mcor 05	MCz	250	YES	62.5
53	Mcor 06	MCz	62.5	YES	125
54	Mcor 07	MCz	I	YES	500
55	Mleu 01	MCz	I	YES	500
56	Mleu 04	MCz	-	YES	250
57	Mleu 05	MCz	500	YES	250
58	Mleu 06	MCz	250	YES	250
59	Mmal 01	MCz	500	YES	250
60	Mmal 03	MCz	250	YES	250
61	Mphi 02	MCz	500	YES	500
62	Mphi 05	MCz	500	YES	>500
63	Mphi 06	MCz	250	YES	250
64	Mphi 09	MCz	250	YES	250
65	Pind 04	MCz	250	YES	250
66	Pind 05	MCz	125	YES	125
67	Pind 06	MCz	125	YES	125
68	Pind 07	MCz	250	YES	I
69	Sgri 01	MCz	250	YES	250
70	Sgri 03	MCz	250	YES	250
71	Sgri 06	MCz	250	YES	250
72	Sgri 07	MCz	500	YES	>500
73	Sgri 08	MCz	I	YES	>500
74	Sher 01	MCz	250	YES	-
75	Sher 03	MCz	500	YES	250
76	Sher 04	MCz	250	YES	250
77	Sher 05	MCz	62.5	YES	250
78	Sher 06	MCz	>500	YES	I
79	Sill 07	MCz	500	YES	500
80	Sind 01	MCz	500	YES	I
81	Sind 02	MCz	500	YES	>500
82	Sind 04	MCz	I	YES	I
83	Sind 06	MCz	I	YES	>500
84	Sind 07	MCz	I	YES	I
85	Sinv 01	MCz	>500	YES	>500

No.	Isolates	Media	MIC (µg/ml)	Media	MIC (µg/ml)
86	Sinv 02	MCz	>500	YES	250
87	Sinv 03	MCz	31.25	YES	62.5
88	Sluz 01	MCz	500	YES	>500
89	Sluz 02	MCz	250	YES	250
90	Sluz 03	MCz	250	YES	500
91	Sluz 05	MCz	250	YES	250
92	Spin 05	MCz	>500	YES	-
93	Tcam 12	MCz	500	YES	250
94	Tcri 02	MCz	500	YES	I
95	Tcri 04	MCz	>500	YES	>500
96	Tcri 06	MCz	250	YES	500
97	Tcri 07	MCz	250	YES	250
98	Tcri 08	MCz	250	YES	500
99	Tint 01	MCz	250	YES	500
100	Tint 02	MCz	250	YES	500
101	Tint 04	MCz	250	YES	500
102	Tpal 01	MCz	250	YES	250
103	Tpal 05	MCz	I	YES	250
104	Ulob 01	MCz	>500	YES	31.25
105	Ulob 04	MCz	I	YES	62.5
106	Uruf 05	MCz	>500	YES	250
107	Uruf 06	MCz	I	YES	I
108	Uruf 08	MCz	I	YES	250
109	Vden 03	MCz	250	YES	62.5
110	Vden 04	MCz	500	YES	500
111	Vden 07	MCz	>500	YES	>500
112	Vden 08	MCz	250	YES	15.63
113	Vden 10	MCz	I	YES	250
114	Vden 11	MCz	250	YES	500
115	Vped 03	MCz	500	YES	>500
116	Vped 05	MCz	>500	YES	500
117	Vped 06	MCz	250	YES	>500
118	Vped 08	MCz	250	YES	500
119	Wtom 01	MCz	>500	YES	I
120	Wtom 02	MCz	500	YES	500
121	Wtom 03	MCz	I	YES	250
122	Wtom 04	MCz	500	YES	500
123	Wtom 05	MCz	500	YES	250

Table 19. Raw data for antimycobacterium activity of mycelium extract extracts from MCz and YES medium

No.	Isolates	Media	MIC (µg/ml)	Media	MIC (µg/ml)
1	Acam 02	MCz	I	YES	I
2	Acam 03	MCz	I	YES	I
3	Acam 04	MCz	I	YES	I
4	Acam 06	MCz	I	YES	I
5	Acam 08	MCz	I	YES	I
6	Acam 09	MCz	I	YES	I
7	Acam 11	MCz	I	YES	I
8	Achi 01	MCz	I	YES	I
9	Achi 02	MCz	I	YES	I
10	Afla 01	MCz	I	YES	I
11	Alan 01	MCz	I	YES	I
12	Alan 02	MCz	I	YES	I
13	Alan 06	MCz	I	YES	I
14	Alan 07	MCz	I	YES	I
15	Amac 02	MCz	I	YES	I
16	Amac 04	MCz	I	YES	I
17	Amac 05	MCz	I	YES	I
18	Amac 09	MCz	I	YES	I
19	Atag 01	MCz	I	YES	I
20	Ccre 01	MCz	I	YES	I
21	Ccre 02	MCz	I	YES	I
22	Ccre 03	MCz	I	YES	I
23	Ccre 04	MCz	I	YES	I
24	Ccre 05	MCz	I	YES	I
25	Ccre 06	MCz	I	YES	I
26	Ccre 09	MCz	I	YES	I
27	Cinc 03	MCz	I	YES	I
28	Cinc 05	MCz	I	YES	I
29	Cpan 03	MCz	I	YES	I
30	Cpan 04	MCz	I	YES	I
31	Cpan 05	MCz	I	YES	I
32	Cpan 06	MCz	I	YES	I
33	Cpan 08	MCz	I	YES	500
34	Cpan 09	MCz	I	YES	I
35	Dfil 03	MCz	I	YES	I
36	Dfil 05	MCz	I	YES	I
37	Dfil 08	MCz	I	YES	I
38	Dfil 09	MCz	I	YES	I
39	Elon 01	MCz	I	YES	I
40	Elon 03	MCz	I	YES	I

No.	Isolates	Media	MIC (µg/ml)	Media	MIC (µg/ml)
41	Esca 02	MCz	I	YES	I
42	Esca 03	MCz	I	YES	I
43	Esca 05	MCz	I	YES	I
44	Haro 03	MCz	I	YES	I
45	Haro 05	MCz	I	YES	I
46	Ijav 02	MCz	I	YES	I
47	Ijav 04	MCz	I	YES	I
48	Ijav 07	MCz	I	YES	I
49	Mcor 01	MCz	I	YES	I
50	Mcor 03	MCz	I	YES	I
51	Mcor 04	MCz	I	YES	I
52	Mcor 05	MCz	I	YES	I
53	Mcor 06	MCz	250	YES	500
54	Mcor 07	MCz	I	YES	I
55	Mleu 01	MCz	I	YES	I
56	Mleu 04	MCz	-	YES	I
57	Mleu 05	MCz	I	YES	I
58	Mleu 06	MCz	I	YES	I
59	Mmal 01	MCz	I	YES	I
60	Mmal 03	MCz	I	YES	I
61	Mphi 02	MCz	I	YES	I
62	Mphi 05	MCz	I	YES	I
63	Mphi 06	MCz	I	YES	I
64	Mphi 09	MCz	I	YES	I
65	Pind 04	MCz	I	YES	I
66	Pind 05	MCz	500	YES	I
67	Pind 06	MCz	250	YES	125
68	Pind 07	MCz	I	YES	I
69	Sgri 01	MCz	I	YES	I
70	Sgri 03	MCz	I	YES	I
71	Sgri 06	MCz	I	YES	I
72	Sgri 07	MCz	I	YES	I
73	Sgri 08	MCz	I	YES	I
74	Sher 01	MCz	-	YES	-
75	Sher 03	MCz	I	YES	I
76	Sher 04	MCz	I	YES	I
77	Sher 05	MCz	I	YES	I
78	Sher 06	MCz	I	YES	I
79	Sill 07	MCz	-	YES	-
80	Sind 01	MCz	I	YES	I
81	Sind 02	MCz	I	YES	I
82	Sind 04	MCz	I	YES	I
83	Sind 06	MCz	I	YES	I

No.	Isolates	Media	MIC (µg/ml)	Media	MIC (µg/ml)
84	Sind 07	MCz	I	YES	I
85	Sinv 01	MCz	I	YES	
86	Sinv 02	MCz	I	YES	I
87	Sinv 03	MCz	I	YES	I
88	Sluz 01	MCz	I	YES	I
89	Sluz 02	MCz	>500	YES	I
90	Sluz 03	MCz	I	YES	I
91	Sluz 05	MCz	I	YES	I
92	Spin 05	MCz	I	YES	-
93	Tcam 12	MCz	I	YES	I
94	Tcri 02	MCz	I	YES	I
95	Tcri 04	MCz	I	YES	I
96	Tcri 06	MCz	I	YES	I
97	Tcri 07	MCz	I	YES	I
98	Tcri 08	MCz	I	YES	I
99	Tint 01	MCz	I	YES	I
100	Tint 02	MCz	I	YES	I
101	Tint 04	MCz	I	YES	I
102	Tpal 01	MCz	I	YES	I
103	Tpal 05	MCz	I	YES	I
104	Ulob 01	MCz	I	YES	I
105	Ulob 04	MCz	I	YES	I
106	Uruf 05	MCz	I	YES	I
107	Uruf 06	MCz	I	YES	I
108	Uruf 08	MCz	I	YES	I
109	Vden 03	MCz	I	YES	I
110	Vden 04	MCz	I	YES	I
111	Vden 07	MCz	I	YES	I
112	Vden 08	MCz	I	YES	I
113	Vden 10	MCz	I	YES	I
114	Vden 11	MCz	I	YES	I
115	Vped 03	MCz	I	YES	-
116	Vped 05	MCz	I	YES	I
117	Vped 06	MCz	I	YES	I
118	Vped 08	MCz	I	YES	I
119	Wtom 01	MCz	I	YES	>500
120	Wtom 02	MCz	I	YES	I
121	Wtom 03	MCz	I	YES	>500
122	Wtom 04	MCz	I	YES	I
123	Wtom 05	MCz	I	YES	250

Table 20. Raw data for antimalarial activity of culture broth extracts from MCz and YES medium.

No.	Isolates	MCz (LC)			YES (LC)		
		500 µg/ml	250 µg/ml	125 µg/ml	500 µg/ml	250 µg/ml	125 µg/ml
1	Acam 02	I	I	I	I	I	I
2	Acam 03	S	S	S	I	I	I
3	Acam 04	S	I	I	S	S	I
4	Acam 06	S	I	I	S	I	I
5	Acam 08	I	I	I	S	I	I
6	Acam 09	I	I	I	I	I	I
7	Acam 11	I	I	I	I	I	I
8	Achi 01	I	I	I	I	I	I
9	Achi 02	I	I	I	S	I	I
10	Afla 01	S	M	I	S	S	I
11	Alan 01	S	I	I	S	I	I
12	Alan 02	S	I	I	I	I	I
13	Alan 06	I	I	I	I	I	I
14	Alan 07	S	I	I	I	I	I
15	Amac 02	S	I	I	S	S	S
16	Amac 04	S	S	S	S	I	I
17	Amac 05	I	I	I	S	I	I
18	Amac 09	I	I	I	S	S	I
19	Atag 01	I	I	I	I	I	I
20	Ccre 01	S	S	M	S	I	I
21	Ccre 02	S	I	I	S	I	I
22	Ccre 03	S	M	I	S	M	I
23	Ccre 04	I	I	I	I	I	I
24	Ccre 05	I	I	I	S	I	I
25	Ccre 06	I	I	I	S	I	I
26	Ccre 09	M	I	I	S	I	I
27	Cinc 03	S	S	S	S	I	I
28	Cinc 05	S	I	I	I	I	I
29	Cpan 03	S	I	I	S	S	S
30	Cpan 04	S	S	S	S	I	I
31	Cpan 05	S	I	I	S	S	S
32	Cpan 06	S	I	I	S	I	I
33	Cpan 08	S	I	I	S	S	I
34	Cpan 09	I	I	I	S	I	I
35	Dfil 03	S	S	S	S	S	S
36	Dfil 05	S	S	S	S	S	I
37	Dfil 08	S	I	I	S	I	I

No.	Isolates	MCz (LC)			YES (LC)		
		500 µg/ml	250 µg/ml	125 µg/ml	500 µg/ml	250 µg/ml	125 µg/ml
38	Dfil 09	S	S	I	S	S	S
39	Elon 01	S	I	I	S	I	I
40	Elon 03	S	S	S	S	S	I
41	Esca 02	S	S	I	S	S	I
42	Esca 03	S	S	S	S	S	S
43	Esca 05	S	S	I	I	I	I
44	Haro 03	S	S	S	S	S	S
45	Haro 05	S	S	S	S	S	S
46	Ijav 02	I	I	I	I	I	I
47	Ijav 04	I	I	I	I	I	I
48	Ijav 07	I	I	I	I	I	I
49	Mcor 01	-	-	-	S	S	I
50	Mcor 03	I	I	I	-	-	-
51	Mcor 04	I	I	I	S	I	I
52	Mcor 05	S	I	I	S	S	S
53	Mcor 06	S	S	S	S	S	S
54	Mcor 07	I	I	I	I	I	I
55	Mleu 01	I	I	I	I	I	I
56	Mleu 04	-	-	-	S	I	I
57	Mleu 05	S	I	I	S	I	I
58	Mleu 06	S	S	S	S	S	I
59	Mmal 01	S	I	I	S	S	S
60	Mmal 03	S	S	I	S	I	I
61	Mphi 02	S	I	I	S	I	I
62	Mphi 05	S	S	I	S	I	I
63	Mphi 06	S	S	S	S	S	I
64	Mphi 09	S	S	S	S	S	M
65	Pind 04	S	S	I	S	I	I
66	Pind 05	S	S	I	S	I	I
67	Pind 06	S	S	I	S	S	I
68	Pind 07	S	S	I	I	I	I
69	Sgri 01	S	S	I	S	I	I
70	Sgri 03	S	S	I	S	S	I
71	Sgri 06	S	I	I	S	S	I
72	Sgri 07	I	I	I	S	I	I
73	Sgri 08	I	I	I	I	I	I
74	Sher 01	I	I	I	-	-	-
75	Sher 03	I	I	I	S	I	I
76	Sher 04	S	I	I	S	S	I

No.	Isolates	MCz (LC)			YES (LC)		
		500 µg/ml	250 µg/ml	125 µg/ml	500 µg/ml	250 µg/ml	125 µg/ml
77	Sher 05	I	I	I	S	I	I
78	Sher 06	I	I	I	I	I	I
79	Sill 07	I	I	I	I	I	I
80	Sind 01	I	I	I	I	I	I
81	Sind 02	S	I	I	I	I	I
82	Sind 04	I	I	I	-	-	-
83	Sind 06	I	I	I	I	I	I
84	Sind 07	I	I	I	I	I	I
85	Sinv 01	I	I	I	I	I	I
86	Sinv 02	I	I	I	S	I	I
87	Sinv 03	I	I	I	S	I	I
88	Sluz 01	I	I	I	I	I	I
89	Sluz 02	I	I	I	I	I	I
90	Sluz 03	S	I	I	S	I	I
91	Sluz 05	S	S	S	I	I	I
92	Spin 05	I	I	I	-	-	-
93	Tcam 12	I	I	I	I	I	I
94	Tcri 02	S	I	I	I	I	I
95	Tcri 04	I	I	I	I	I	I
96	Tcri 06	S	S	I	I	I	I
97	Tcri 07	S	S	I	S	S	I
98	Tcri 08	S	S	I	I	I	I
99	Tint 01	I	I	I	I	I	I
100	Tint 02	S	I	I	I	I	I
101	Tint 04	I	I	I	I	I	I
102	Tpal 01	I	I	I	S	I	I
103	Tpal 05	I	I	I	S	I	I
104	Ulob 01	I	I	I	I	I	I
105	Ulob 04	I	I	I	I	I	I
106	Uruf 05	I	I	I	I	I	I
107	Uruf 06	M	I	I	I	I	I
108	Uruf 08	I	I	I	I	I	I
109	Vden 03	S	S	I	S	S	I
110	Vden 04	I	I	I	I	I	I
111	Vden 07	I	I	I	S	I	I
112	Vden 08	S	S	M	S	S	S
113	Vden 10	I	I	I	S	S	S
114	Vden 11	S	S	M	S	S	I

No.	Isolates	MCz (LC)			YES (LC)		
		500 µg/ml	250 µg/ml	125 µg/ml	500 µg/ml	250 µg/ml	125 µg/ml
115	Vped 03	S	S	M	-	-	-
116	Vped 05	S	I	I	I	I	I
117	Vped 06	S	S	S	S	I	I
118	Vped 08	S	S	S	S	I	I
119	Wtom 01	I	I	I	S	I	I
120	Wtom 02	M	I	I	I	I	I
121	Wtom 03	I	I	I	S	S	M
122	Wtom 04	S	I	I	S	S	I
123	Wtom 05	S	I	I	S	S	S

Table 21. Raw data for antimalarial activity of mycelium extracts from MCz and YES medium.

No.	Isolates	MCz (LC)			YES (LC)		
		500 µg/ml	250 µg/ml	125 µg/ml	500 µg/ml	250 µg/ml	125 µg/ml
1	Acam 02	I	I	I	I	I	I
2	Acam 03	I	I	I	I	I	I
3	Acam 04	I	I	I	I	I	I
4	Acam 06	I	I	I	I	I	I
5	Acam 08	I	I	I	I	I	I
6	Acam 09	I	I	I	I	I	I
7	Acam 11	I	I	I	I	I	I
8	Achi 01	S	I	I	I	I	I
9	Achi 02	I	I	I	I	I	I
10	Afla 01	I	I	I	I	I	I
11	Alan 01	I	I	I	I	I	I
12	Alan 02	I	I	I	I	I	I
13	Alan 06	S	I	I	I	I	I
14	Alan 07	I	I	I	I	I	I
15	Amac 02	I	I	I	I	I	I
16	Amac 04	I	I	I	I	I	I
17	Amac 05	I	I	I	I	I	I
18	Amac 09	I	I	I	I	I	I
19	Atag 01	I	I	I	I	I	I
20	Ccre 01	I	I	I	I	I	I
21	Ccre 02	I	I	I	I	I	I
22	Ccre 03	I	I	I	I	I	I
23	Ccre 04	I	I	I	I	I	I

No.	Isolates	MCz (LC)			YES (LC)		
		500 µg/ml	250 µg/ml	125 µg/ml	500 µg/ml	250 µg/ml	125 µg/ml
24	Ccre 05	I	I	I	I	I	I
25	Ccre 06	I	I	I	I	I	I
26	Ccre 09	I	I	I	I	I	I
27	Cinc 03	I	I	I	I	I	I
28	Cinc 05	I	I	I	I	I	I
29	Cpan 03	I	I	I	I	I	I
30	Cpan 04	I	I	I	I	I	I
31	Cpan 05	I	I	I	I	I	I
32	Cpan 06	I	I	I	I	I	I
33	Cpan 08	I	I	I	I	I	I
34	Cpan 09	I	I	I	I	I	I
35	Dfil 03	I	I	I	I	I	I
36	Dfil 05	I	I	I	I	I	I
37	Dfil 08	I	I	I	I	I	I
38	Dfil 09	I	I	I	I	I	I
39	Elon 01	I	I	I	I	I	I
40	Elon 03	I	I	I	I	I	I
41	Esca 02	I	I	I	I	I	I
42	Esca 03	I	I	I	I	I	I
43	Esca 05	I	I	I	I	I	I
44	Haro 03	S	S	I	I	I	I
45	Haro 05	I	I	I	I	I	I
46	Ijav 02	I	I	I	I	I	I
47	Ijav 04	I	I	I	I	I	I
48	Ijav 07	I	I	I	I	I	I
49	Mcor 01	I	I	I	I	I	I
50	Mcor 03	I	I	I	I	I	I
51	Mcor 04	I	I	I	I	I	I
52	Mcor 05	I	I	I	S	I	I
53	Mcor 06	S	S	I	S	I	I
54	Mcor 07	I	I	I	I	I	I
55	Mleu 01	I	I	I	I	I	I
56	Mleu 04	-	-	-	I	I	I
57	Mleu 05	I	I	I	I	I	I
58	Mleu 06	I	I	I	I	I	I
59	Mmal 01	I	I	I	I	I	I
60	Mmal 03	I	I	I	I	I	I
61	Mphi 02	I	I	I	S	I	I
62	Mphi 05	S	I	I	I	I	I
63	Mphi 06	I	I	I	S	I	I
64	Mphi 09	I	I	I	I	I	I

No.	Isolates	MCz (LC)			YES (LC)		
		500 µg/ml	250 µg/ml	125 µg/ml	500 µg/ml	250 µg/ml	125 µg/ml
65	Pind 04	S	I	I	I	I	I
66	Pind 05	I	I	I	I	I	I
67	Pind 06	I	I	I	I	I	I
68	Pind 07	I	I	I	I	I	I
69	Sgri 01	I	I	I	I	I	I
70	Sgri 03	I	I	I	I	I	I
71	Sgri 06	I	I	I	I	I	I
72	Sgri 07	I	I	I	I	I	I
73	Sgri 08	I	I	I	I	I	I
74	Sher 01	-	-	-	-	-	-
75	Sher 03	I	I	I	I	I	I
76	Sher 04	S	I	I	S	I	I
77	Sher 05	I	I	I	I	I	I
78	Sher 06	I	I	I	I	I	I
79	Sill 07	-	-	-	-	-	-
80	Sind 01	I	I	I	I	I	I
81	Sind 02	I	I	I	I	I	I
82	Sind 04	I	I	I	I	I	I
83	Sind 06	I	I	I	I	I	I
84	Sind 07	I	I	I	I	I	I
85	Sinv 01	I	I	I	I	I	I
86	Sinv 02	I	I	I	I	I	I
87	Sinv 03	I	I	I	I	I	I
88	Sluz 01	I	I	I	I	I	I
89	Sluz 02	I	I	I	I	I	I
90	Sluz 03	I	I	I	I	I	I
91	Sluz 05	I	I	I	I	I	I
92	Spin 05	I	I	I	I	I	I
93	Tcam 12	I	I	I	I	I	I
94	Tcri 02	I	I	I	I	I	I
95	Tcri 04	I	I	I	I	I	I
96	Tcri 06	I	I	I	I	I	I
97	Tcri 07	I	I	I	I	I	I
98	Tcri 08	I	I	I	I	I	I
99	Tint 01	I	I	I	I	I	I
100	Tint 02	I	I	I	I	I	I
101	Tint 04	I	I	I	I	I	I
102	Tpal 01	I	I	I	I	I	I

No.	Isolates	MCz (LC)			YES (LC)		
		500 µg/ml	250 µg/ml	125 µg/ml	500 µg/ml	250 µg/ml	125 µg/ml
103	Tpal 05	I	I	I	I	I	I
104	Ulob 01	I	I	I	I	I	I
105	Ulob 04	I	I	I	I	I	I
106	Uruf 05	I	I	I	I	I	I
107	Uruf 06	I	I	I	I	I	I
108	Uruf 08	I	I	I	I	I	I
109	Vden 03	I	I	I	I	I	I
110	Vden 04	I	I	I	I	I	I
111	Vden 07	I	I	I	I	I	I
112	Vden 08	I	I	I	I	I	I
113	Vden 10	I	I	I	I	I	I
114	Vden 11	I	I	I	I	I	I
115	Vped 03	I	I	I	I	I	I
116	Vped 05	I	I	I	I	I	I
117	Vped 06	I	I	I	I	I	I
118	Vped 08	I	I	I	I	I	I
119	Wtom 01	I	I	I	I	I	I
120	Wtom 02	I	I	I	I	I	I
121	Wtom 03	I	I	I	I	I	I
122	Wtom 04	I	I	I	I	I	
123	Wtom 05	I	I	I	I	I	

I = Inactive

M = Moderately active

S = Strongly active

SA = *S. aureus* EF = *E. faecalis* BS = *B. subtilis*

CE = *E. coli* PA = *P. aeruginosa* CA = *C. albicans*

TM = *T. mentagrophytes*

BIOGRAPHY

NAME	Mr. Wattana Panphut
DATE OF BIRTH	5 August 1970
PLACE OF BIRTH	Muang District, Kanchanaburi Province, Thailand
INSTITUTION ATTENED	King Mongkut's Institute of Technology Thonburi, 1993-1996: Bachelor of Science (Microbiology)
FELLOWSHIP/ RESEARCH GRANT	Institutional Strengthening Program Grant from the National Science and Technology Development Agency (NSTDA), 1997-1998. Partial support by Biodiversity Research & Training Program (BRT 542104), 1999 Supported in part by The Thesis Grant, Faculty of Graduate Studies, Mahidol University, 2000