

ลักษณะสมบัติของ *Aspergillus* spp. สายพันธุ์ที่สร้างและไม่สร้างสารพิษที่คัดแยกจากไร้  
ข้าวโพดในประเทศไทย



นายขรรค์ชัย ดันเมฆ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต

สาขาวิชาเทคโนโลยีชีวภาพ

คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

ปีการศึกษา 2551

ลิขสิทธิ์ของจุฬาลงกรณ์มหาวิทยาลัย

CHARACTERIZATION OF TOXIGENIC AND ATOXIGENIC *Aspergillus* spp.  
STRAINS ISOLATED FROM CORN FIELDS IN THAILAND

Mr. Khanchai Danmek

A Dissertation Submitted in Partial Fulfillment of the Requirements  
for the Degree of Doctor of Philosophy Program in Biotechnology

Faculty of Science

Chulalongkorn University

Academic Year 2008

Copyright of Chulalongkorn University

512162



บรรณานุกรม : ลักษณะสมบัติของ *Aspergillus* spp. สายพันธุ์ที่สร้างและไม่สร้างสารพิษที่คัดแยกจากไร่ข้าวโพดในประเทศไทย. (CHARACTERIZATION OF TOXIGENIC AND ATOXIGENIC *Aspergillus* spp. STRAINS ISOLATED FROM CORN FIELDS IN THAILAND) อ.ที่ปรึกษาวิทยานิพนธ์หลัก : รศ. ดร. ھرรรร รุณณะพัคค์ม, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม PROF. Kenneth E. Damann, Jr.Ph.D. 179 หน้า.

*Aspergillus flavus* มีลักษณะทางสัณฐานวิทยาคล้ายกับเชื้อราชนิดอื่นๆ ใน section *Flavi* เชื้อรานี้สามารถจำแนกออกจากตัวอื่นๆ โดยอาศัยลักษณะทางสัณฐานวิทยา จากการศึกษา *A. flavus* สายพันธุ์ต่างๆ เช่น *A. flavus* NRRL 3357, NRRL 21882, TX9-8, F3W4, Af53 และ *A. flavus* ที่คัดแยกจากดินบริเวณที่ปลูกข้าวโพดในประเทศไทย และชนิดอื่นๆ ใน section *Flavi* เช่น *A. parasiticus* *A. tamarisii* และ *A. nomius* พบว่ามีเพียง *A. flavus* ที่สามารถผลิต synnema เมื่อเลี้ยงในอาหารสูตร Czapek Dox ที่มีการเติม Avid® ซึ่งวิธีการนี้สามารถช่วยในการจำแนกเชื้อรา *A. flavus* ออกจากสายพันธุ์อื่นๆ ที่มีลักษณะคล้ายกัน ใน section *Flavi* ได้ เช่น *A. parasiticus* *A. tamarisii* และ *A. nomius* เชื้อรา *A. flavus* และสายพันธุ์อื่นๆ ใน section *Flavi* ได้ถูกคัดแยก และศึกษาลักษณะสมบัติจากดินบริเวณที่มีการปลูกข้าวโพดในประเทศไทยจากการศึกษาพบว่า *Aspergillus* section *Flavi* มีการสร้างสีที่ด้านล่างโคโลนีเมื่อเลี้ยงบนอาหารสูตร *Aspergillus* *Flavus* and *Parasiticus* Agar (AFPA) ที่อุณหภูมิ 30°C เป็นระยะเวลา 7 วันในที่มืด เช่น สีเหลืองส้ม (*A. flavus* *A. parasiticus* และ *A. oryzae*) สีเหลืองจาง (*A. parasiticus* และ *A. nomius*) และสีน้ำตาลจางถึงเข้ม (*A. tamarisii* และ *A. pseudotamarisii*) มีเพียง *A. flavus* ที่สร้าง conidial head ที่มีการเรียงของ conidium เป็นรูปกระสวยในอาหารสูตร V8 medium อย่างไรก็ตาม *Aspergillus* section *Flavi* ทุกสายพันธุ์มีการผลิต Kojic acid ในอาหารสูตร modified rice medium และเมื่อศึกษาถึงการผลิต aflatoxin พบว่าเชื้อราที่สามารถผลิตได้คือ *A. flavus* *A. parasiticus* และ *A. nomius* แต่จะมีปริมาณต่างกัน เช่น *A. parasiticus* ผลิต aflatoxin B มากกว่า ในขณะที่ *A. nomius* ผลิต aflatoxin G มากกว่า B และ *A. flavus* สามารถพบได้ตั้งแต่สายพันธุ์ที่ไม่ผลิต จนถึงสายพันธุ์ที่ผลิต aflatoxin ในปริมาณมาก ในการศึกษาสามารถพบเชื้อรา *A. flavus* ที่ไม่ผลิต aflatoxin จำนวน 33 สายพันธุ์

สาขาวิชา เทคโนโลยีชีวภาพ.....

ปีการศึกษา 2551.....

ลายมือชื่อนิสิต.....

ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์ร่วม.....

## 4773801123 : MAJOR BIOTECHNOLOGY


KEYWORDS : ASPERGILLUS FLAVUS / AFLATOXINS / CORN / TOXIGENIC / ATOXIGENIC


KHANCHAI DANMEK : CHARACTERIZATION OF TOXIGENIC AND ATOXIGENIC *Aspergillus* spp. STRAINS ISOLATED FROM CORN FIELDS IN THAILAND. ADVISOR : ASSOC. PROF. Hunsa Punnapayak, Ph.D., CO-ADVISOR : PROF. Kenneth E. Damann, Jr, Ph.D., 179 pp.

*Aspergillus flavus* is morphologically similar to other species of the section *Flavi*. A facile method to distinguish these species based on cultural characteristics is reported. Several *A. flavus* and some related strains in section *Flavi* including *A. parasiticus*, *A. nomius*, and *A. tamarii* were isolated, and characterized from corn fields in different areas of Thailand. *Aspergillus* section *Flavi* produced a wide range of color in reverse on *Aspergillus flavus* and *parasiticus* agar (AFPA) at 30 °C for 7 days in the dark including dark yellow orange (*A. flavus*, *A. parasiticus*, and *A. oryzae*), pale yellow (*A. parasiticus*, and *A. nomius*), and pale or dark brown (*A. tamarii*, and *A. pseudotamarii*). Only *A. flavus* produced columnar shape of conidiophores in V8 medium. All isolated strains in *Aspergillus* section *Flavi* produced kojic acid in modified rice medium. Only *A. flavus* produced synnemata on modified Czapek medium containing (20-1,000 μl/L) Avid®. This novel, yet readily prepared, culture medium permits differentiation of *A. flavus* from related species in section *Flavi*. Aflatoxins producing strains were identified to be *A. flavus*, *A. parasiticus*, and *A. nomius*. Isolated fungi in *Aspergillus* section *Flavi* differently produced aflatoxins including *A. parasiticus* produced aflatoxin B more than G, *A. nomius* produced aflatoxin G more than B, and *A. flavus* widely produced range of aflatoxins. Twenty three stains of atoxigenic strains of *A. flavus* were observed in the isolates. Some aflatoxin producing strains of *A. flavus* were obtained from some areas without more corn cultivation, produced high quantity of aflatoxin yields. So high level of corn cultivation areas did not specific to find aflatoxin producing strains of *A. flavus* in Thailand. Inhibition of aflatoxins production from toxigenic strains *A. flavus* by the same strain of atoxigenic involves solution factors more than living cell of fungi (mycelial network, or touching).

Field of Study : Biotechnology.....

Academic Year : 2008.....

Student's Signature : 

Advisor's Signature : 

Co-advisor's Signature : 

## ACKNOWLEDGEMENT

I would like to acknowledge my heartfelt gratitude and appreciation to Assoc. Dr. Hunsu Punnapayak, my advisor and Prof. Dr. Kenneth E. Damann, Jr., my co-advisor, for the support and invaluable guidance given throughout the course of this study. I am indebted to them for their kind supervision, invaluable guidance, constant encouragement review and correction of the dissertation content

I am grateful to Assoc. Prof. Dr. Prada Boon-lung, Prof. Micheal Bravermann, Assoc. Prof. Dr. Sirirat Rengpipat, Assist Prof. Dr. Pongtarin Lotrakul, and Dr. Sehanat Prasongsuk, for useful comments and critical reading of this dissertation.

I express my warm thanks to Plant Biomass Utilization Research Unit, Department of Botany, Faculty of Science, Biotechnology Program, Faculty of Science, Chulalongkorn University and Agcenter, Mycotoxicology Laboratory, Department of Plant Pathology and Crop Physiology, Louisiana State University, for partly financing for this study and providing facilities throughout the research.

I express my gratitude to my friends and the personnel in Department of Botany and Biotechnology Program, Faculty of Science, Chulalongkorn University for providing excellent working facilities and a friendly atmosphere.

Finally, I would like to express my deepest appreciation to my family for their love, encouragement and worthy moral support throughout my study at Chulalongkorn University.

# CONTENTS

	Page
ABSTRACT (IN THAI).....	iv
ABSTRACT (IN ENGLISH).....	v
ACKNOWLEDGEMENTS.....	vi
CONTENT.....	vii
LIST OF TABLES.....	x
LIST OF FIGURES.....	xii
CHAPTER I INTRODUCTION.....	1
CHAPTER II THEORY AND LITERATURE REVIEWS.....	3
2.1 Economic significance of aflatoxin contamination.....	3
2.2 <i>Aspergillus</i> section <i>Flavi</i> ; Major source of aflatoxin producing fungi.....	5
2.3 Morphological Studies of aflatoxin producing fungi.....	8
2.4 Major metabolites products from <i>Aspergillus</i> section <i>Flavi</i> .....	10
2.4.1 Primary metabolites: Extracellular enzymes and their application.....	10
2.4.1.1 Amylolytic enzymes.....	11
2.4.1.2 Proteolytic enzymes.....	12
2.4.1.3 Cellulolytic enzymes.....	12
2.4.2 Secondary metabolites.....	13
2.4.2.1 Cyclopiazonid acid (CPA).....	13
2.4.2.2 Kojic acid.....	14
2.4.2.3 Aflatoxin.....	14
2.5 Biosynthesis of aflatoxin.....	15
2.6 Environmental and nutritional factors affecting aflatoxin biosynthesis....	21
2.6.1 Carbons affecting.....	21
2.6.2 Nitrogen affecting.....	23
2.6.3 pH affecting.....	24
2.7 Isolation of aflatoxin producing fungi.....	24
2.8 Biocontrol of aflatoxin producing strains with atoxigenic strains of <i>A. flavus</i> .....	25

	<b>Page</b>
CHAPTER III EXPERIMENTAL.....	27
3.1 Materials.....	27
3.1.1 Laboratory Facilities and Equipment.....	27
3.1.2 Chemicals.....	28
3.1.3 Isolates.....	29
3.2 Collection of the soil samples from different corn fields of Thailand.....	30
3.3 Morphological observation on <i>A. flavus</i> and some related strains in section <i>Flavi</i> .....	31
3.3.1 <i>A. flavus</i> isolation.....	32
3.3.2 Fungal spore preparation from a single colony <i>A. flavus</i> .....	33
3.3.3 <i>A. flavus</i> identification by colony observation.....	33
3.3.4 Effect of different surfactants types on fungal colonization.....	33
3.4 Identification of <i>A. flavus</i> by synnemata formation on Czapek Dox agar containing Avid <sup>®</sup> .....	34
3.4.1 Effect of light and dark on synnemata formation by <i>A. flavus</i> ...	34
3.4.2 Effect of media types on synnemata formation by <i>A. flavus</i> .....	35
3.4.3 Effect of Avid <sup>®</sup> concentration on synnemata formation by <i>A. flavus</i> .....	35
3.5 Determining Vegetative compatibility groups (VCGs) of <i>A. flavus</i> .....	36
3.5.1 <i>Nit</i> mutant selection.....	36
3.5.2 Nitrate-nonutilizing ( <i>nit</i> ) mutant selection.....	37
3.5.3 Complementation between different types ( <i>niaD</i> , <i>nirA</i> , and <i>cnx</i> ) of <i>nit</i> mutants.....	37
3.5.3.1 Spore suspension.....	37
3.5.3.2 Complementation between different types ( <i>niaD</i> , <i>nirA</i> , and <i>cnx</i> ) of <i>nit</i> mutants.....	37
3.6 Toxic and some metabolite production and detection.....	38



	<b>Page</b>
3.7 Inhibition of aflatoxins production from toxigenic strains of <i>A. flavus</i> by the same strain of atoxigenic.....	39
3.8 Statistical Analysis.....	40
CHAPTER IV RESULTS.....	46
4.1 Collection of the soil samples from different corn fields of Thailand.....	47
4.2 Morphological observation on <i>A. flavus</i> and some related strains in section <i>Flavi</i> .....	48
4.3 Distribution of <i>Aspergillus</i> section <i>Flavi</i> from corn fields in Thailand.....	60
4.4 Determining Vegetative compatibility groups (VCGs) of <i>A. flavus</i> .....	94
4.5 Complementation between different types ( <i>niaD</i> , <i>nirA</i> , and <i>cnx</i> ) of <i>nit</i> mutants.....	98
4.6 Toxic and some metabolite production and detection.....	111
4.7 Inhibition of aflatoxins from toxigenic strains of <i>A. flavus</i> .....	131
CHAPTER V DISCUSSION AND CONCLUSIONS.....	139
REFERENCES.....	152
APPENDIXES.....	174
BIOGRAPHY.....	179

## LIST OF TABLES

Table No.	page
2.1	Morphological separation of <i>A. flavus</i> and <i>A. parasiticus</i> .....9
4.1	Soil samples were collected from Northern Thailand.....48
4.2	Soil samples were collected from Northeastern Thailand .....49
4.3	Soil samples were collected from central Thailand.....49
4.4	Isolated strains of <i>Aspergillus</i> section <i>Flavi</i> from corn fields in Thailand cultivated on AFPA medium at 30 °C for 7 days.....50
4.5	Distribution of <i>Aspergillus</i> section <i>Flavi</i> from corn fields in Thailand.....80
4.6	Vegetative compatibility groups of isolated <i>A. flavus</i> from different geographic areas of corn fields in Thailand.....85
4.7	Lists of proteolytic producing strains (showed zone of clearance) of isolates <i>Aspergillus</i> .....88
4.8	Lists of amylolytic producing strains (showed zone of clearance) of isolates <i>Aspergillus</i> .....89
4.9	Lists of cellulolytic producing strains (showed zone of clearance) of isolates <i>Aspergillus</i> .....90
4.10	Number of <i>Aspergillus</i> had ability to use Citrate utilizing medium and change into blue color at 30 °C and 2 days in dark.....91
4.11	number of <i>Aspergillus</i> had/ had not ability to change Cz medium to brown when incubated at 30 °C for 7 days in the dark.....94
4.12	Aflatoxins production from <i>A. ochraceus/melleus</i> Danmek 098-102 BCU in mYES at 30°C for 7 days.....97
4.13	Aflatoxins production from <i>A. tamarii</i> Danmek 080-097 BCU in mYES at 30°C for 7 days.....97
4.14	Aflatoxins production from <i>A. nomius</i> Danmek 068-079 BCU in mYES at 30°C for 7 days.....98
4.15	Aflatoxins production from <i>A. parasiticus</i> Danmek 056-067 BCU in mYES at 30°C for 7 days.....99

<b>Table No.</b>	<b>page</b>
4.16 Aflatoxins production from <i>A. flavus</i> Danmek 001-055 and 103-256 BCU in mYES at 30°C for 7 days.....	99
4.17 aflatoxin production datas from isolated strains of <i>Aspergillus</i> section <i>Flavi</i> from Thailand.....	107

## LIST OF FIGURES

Figure No.	Page
2.1	Cyclopiazonic acid.....13
2.2	Kojic acid.....14
2.3	The structure of aflatoxins: B1, B2, G1, G2.....15
2.4	The gene cluster responsible for aflatoxin biosynthesis in <i>Aspergillus flavus</i> and <i>A. parasiticus</i> .....17
4.1	corn fields, and sampling areas in Thailand (2005).....48
4.2	Isolated strains of <i>Aspergillus</i> section <i>Flavi</i> from corn fields in Thailand cultivated on AFPA medium at 30 °C for 7 days.....52
4.3	standard strains of <i>Aspergillus</i> section <i>Flavi</i> on AFPA medium at 30 °C for 7 days.....53
4.4	Single sporulation of <i>Aspergillus</i> on 5% V8 agar pH 5.5 at 30 °C in the dar.....54
4.5	<i>A. tamaris</i> on AFPA, Cz, and V8 medium at 30 °C in the dark for 7 day.....56
4.6	<i>A. parasiticus/sojae</i> on AFPA, Cz, and V8 medium at 30 °C in the dark for 7 days.....57
4.7	<i>A. nomius</i> on AFPA, Cz, and V8 medium at 30 °C in the dark for 7 days.....58
4.8	<i>A. flavus</i> on AFPA, Cz, and V8 medium at 30 °C in the dark for 7 days.....59
4.9	<i>A. melleus/ochraceus</i> on AFPA, Cz, and V8 medium at 30 °C in the dark for 7 days.....60
4.10	standard strains of <i>Aspergillus</i> section <i>Flavi</i> on AFPA, Cz, and V8 medium at 30 °C for 7 days.....61
4.11	droplets formation in <i>A. nomius</i> on Cz at 30 °C.....62
4.12	<i>Aspergillus</i> on 5%V8 agar at 30 °C for 14 days.....63
4.13	<i>Aspergillus</i> sclerotia.....64
4.14	<i>A. flavus</i> conidiophores on different types of media at 30 °C for 7 days in the dark.....65

Figure No.	Page
4.15 standard strains of <i>Aspergillus</i> section <i>Flavi</i> on different type media at 30 °C for 7 days.....	66
4.16 Isolated strains of <i>Aspergillus</i> section <i>Flavi</i> incubated in Czapek medium with or without Avid® at 30 °C in the dark.....	68
4.17 Standard <i>Aspergillus</i> section <i>Flavi</i> (NRRL, and SRRC, USA) incubated in Czapek medium without Avid® .....	69
4.18 different characteristic of synnemata producing strains of <i>A. flavus</i> .....	69
4.19 Morphological structures of <i>A. flavus</i> on modified Cz medium containing Avid® at 30 °C in the dark for 10 days.....	70
4.20 Isolated strains of <i>Aspergillus</i> section <i>Flavi</i> incubated in Czapek medium with or without Avid® at 30 °C in the dark.....	71
4.21 isolated strains of <i>A. flavus</i> on different media types with or without 40 µl/L of Avid® at 30 °C for 7 days in the dark.....	73
4.22 <i>A. flavus</i> incubated in Czapek medium with or without Avid®, and replacing sodium nitrate with either ammonium sulphate, ammonium tartrate, or peptone at 30 °C in the dark for 7 days.....	75
4.23 <i>A. tamarii</i> on Cz medium containing difference Avid® concentration in the dark at 30 °C for 7 days.....	76
4.24 <i>A. nomius</i> on Cz medium containing different Avid® concentration in the dark at 30 °C for 7 days.....	77
4.25 <i>A. parasiticus</i> on Cz medium containing different Avid® concentration in the dark at 30 °C for 7 days.....	78
4.26 <i>A. flavus</i> on Cz medium containing different Avid® concentration in the dark at 30 °C for 7 days.....	79
4.27 <i>A. flavus</i> on Rose Bengal-chlorate medium at 30 °C for 10 days in the dark .....	82

Figure No.	Page
4.28	<i>Nit</i> mutants of <i>A. flavus</i> on Rose Bengal-chlorate medium at 30 °C for 10 days in the dark for 10 days.....82
4.29	<i>niaD</i> , <i>nirA</i> , and <i>cnx</i> mutant of <i>A. flavus</i> on Cz containing each ammonium tartrate, nitrite, and hypoxanthine as sole nitrogen sources.....83
4.30	Complementation between different types of fungal strains.....84
4.31	Distribution of seventeen VCGs of <i>A. flavus</i> .....86
4.32	<i>Aspergillus</i> Cz medium containing skim milk as the sole carbon source, and incubated at 30 °C for 3 to 4 days.....87
4.33	<i>Aspergillus</i> Cz medium containing potato starch as the sole carbon source, and incubated at 30 °C for 3 to 4 days.....88
4.34	<i>Aspergillus</i> Cz medium containing CMC as the sole carbon source, and incubated at 30 °C for 3 to 4 days.....90
4.35	<i>Aspergillus</i> in Citrate utilizing medium at 30 °C and 2 days in dark.....92
4.36	<i>Aspergillus</i> in Rice medium and incubated at 30 °C for 7 days in the dark.....93
4.37	<i>Aspergillus</i> in Cz medium and incubated at 30 °C for 7 days in the dark.....94
4.38	extracted solution from Rice medium.....95
4.39	Aflatoxins detection on TLC plate under longwave UV light.....96
4.40	Distribution of aflatoxins producing strains of <i>A. flavus</i> from different geographic corn fields in Thailand.....109
4.41	<i>Aspergillus</i> colonization in mYES at 30°C, in the dark for 7 days.....109
4.42	Co-inoculation of <i>A. flavus</i> NRRL 3357 with each isolated atoxigenic <i>A. flavus</i> from Thailand in mYES at 30°C, in the dark for 7 days.....110
4.43	aflatoxin B1 production from <i>A. flavus</i> NRRL 3357 and co-inoculation of <i>A. flavus</i> NRRL 3357 with atoxigenic <i>A. flavus</i> Danmek 003 BCU in mYES at 30°C, in the dark for 7 days.....111

<b>Table No.</b>	<b>page</b>
4.44 aflatoxin B1 production from <i>A. flavus</i> NRRL 3357 and addition timing of atoxigenic <i>A. flavus</i> Danmek 003 BCU culture in mYES at 30 <sup>o</sup> C, in the dark for 7 days.....	112
4.45 Aflatoxin yield by co-inoculation (50:50 of 150 µl of 10 <sup>8</sup> spores) of <i>A. flavus</i> NRRL 3357 with <i>A. flavus</i> Danmek 003 BCU in 10 ml YES medium in the dark at 30 <sup>o</sup> C.....	113
4.46 Aflatoxin B1 from growing together or separated between <i>A. flavus</i> NRRL 3357 with <i>A. flavus</i> Danmek 003 BCU in mYES medium at 30 <sup>o</sup> C.....	115
4.47 <i>Aspergillus</i> colonization in mYES at 30 <sup>o</sup> C in the dark for 3 days	
4.48 Aflatoxin B1 from <i>Aspergillus</i> culture in mYES medium with or without gallic acid.....	115