STUDIES ON PATHOGENICITY OF LOCAL ISOLATE OF *NOMURAEA RILEYI* AGAINST *SPODOPTERA LITURA*

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Studies on pathogenesis of the fungal isolate, *N. rileyi* against *Spodoptera litura* were carried out through the examination of thin sections of infected larvae under a light microscope. The conidia of fungal isolate germinated on the larval integument of *S. litura* ca. 20 to 24 h and penetrated the cuticle within 48 h. The invasion hyphae converted to hyphal bodies after reaching the haemocoel. The multiplication of hyphal bodies occurred throughout all tissues of insect larvae within 6 to 6.5 days after inoculation. Noninvasion hyphal bodies converted to invasion mycelia. The conidiophores emerged from insect cadavers, were observed at 7 days after inoculation, and subsequently whole cadavers were completely covered by green conidia. The complete development cycle of *N. rileyi* in *S. litura* larvae was accomplished in approximately 8 to 9 days. Attempts to determine effects of culture media on the virulence of *N. rileyi* were carried out. The 4th passage of serial transfer of conidia on SMAY medium, whose ability to produce yeast-liked hyphal bodies was lost, exhibited the reduction in virulence against larvae of *S. litura*. In addition, the deficiency in sporulation of these conidia on insect cadavers was observed after the 8th passage onward as opposed to those conidia derived from FMAY medium that could sustain their virulence and spore production. It was found that the major fatty acid compositions of fish soluble extract, which were palmitic acid and oleic acids, could induce the germination of conidia and spore production of *N. rileyi*. The conidia derived from FMAY medium retained their capability of germination, so that the germination of conidia was more rapid than those derived from SMAY medium. Protein patterns expressed during the germination of conidia were compared with the virulent and attenuated strains, using SDS-PAGE. The virulent strains displayed the additional two distinct bands from those of attenuated strains. These unknown extra bands were found to have molecular weights of approximately 43 and 25.04 kDa, while the structure of proteins was still unknown. Studies on the relationship of cuticle-degrading enzymes to the virulence of *N. rileyi* suggested that the protease was probably the important factor in penetration of the larval integument, while the other cuticle-degrading enzymes, i.e., chitinases and lipases, were presumably essential for the development of *N. rileyi*.

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พรพจน์ ศรีสุขชยะกุล : การศึกษาการก่อโรคของเชื้อรา *Nomuraea rileyi* ที่แยกได้ภายในประเทศต่อหนอนกระทู้ผัก (STUDIES ON PATHOGENICITY OF LOCAL ISOLATE OF *NOMURAEA RILEYI* AGAINST *SPODOPTERA LITURA*.). คณะกรรมการควบคุมวิทยานิพนธ์ : สมศักดิ์ พันธุวัฒนา, Ph.D., อมเรศ ภูมิรัตน, Ph.D., จันทร์เพ็ญ วิวัฒน์, Ph.D. 103 หน้า. ISBN 974-04-2482-1

จากการศึกษาถึงปัจจัยต่างๆ ที่มีผลต่อการก่อโรคของเชื้อรา *Nomuraea rileyi* ในหนอนกระทู้ผัก *Spodoptera litura* นั้นพบว่าสปอร์ส่วนใหญ่ของเชื้อราจะใช้เวลาในการงอกประมาณ 20 ถึง 24 ชั่วโมง โดย germ tube จะแทงผ่านผนังลำตัวของหนอนภายในระยะเวลา 2 วันหลังจากที่ได้รับเชื้อ เส้นใยของเชื้อราจะแทงเข้าสู่ haemocoel จากนั้นเส้นใยของเชื้อราก็จะเริ่มเข้าสู่ระยะการเจริญเติบโตแบบยีสต์ และต่อมาอีกประมาณ 3 วัน ยีสต์จะเปลี่ยนรูปร่างกลับไปเป็นเส้นใยอีกครั้งหนึ่ง เพื่อแทงทะลุออกจากผนังลำตัวของหนอน เส้นใยสีขาวจะเจริญแผ่กระจายปกคลุมตัวหนอนแล้วสร้างสปอร์สีเขียว ซึ่งกระบวนการเหล่านี้จะใช้เวลาทั้งสิ้นประมาณ 8-9 วันหลังจากที่ได้รับเชื้อ จากการศึกษาถึงองค์ประกอบของอาหารเลี้ยงเชื้อที่อาจมีผลต่อความสามารถของเชื้อราในการฆ่าหนอนพบว่า อาหารเลี้ยงเชื้อสูตร SMAY มีผลต่อการสร้างสปอร์ของเชื้อราโดยทำให้เชื้อราสร้างสปอร์ลดลง และอาหารเลี้ยงเชื้อสูตรนี้ยังมีผลทำให้ความสามารถในการฆ่าหนอนของเชื้อราลดลงหลังจากที่ได้ทำการเลี้ยงเชื้อบนอาหารชนิดนี้เป็นระยะเวลานานอีกด้วย ในขณะที่อาหารเลี้ยงเชื้อสูตร FMAY ให้ผลในทางตรงกันข้าม ซึ่งเป็นผลมาจาก ส่วนประกอบของกรดไขมันที่มีอยู่ในน้ำสกัดจากปลา ซึ่งใช้เป็นส่วนประกอบของอาหารเลี้ยงเชื้อสูตร FMAY โดยพบว่า ส่วนประกอบดังกล่าวมีบทบาทสำคัญเกี่ยวข้องกับการงอกและการสร้างสปอร์ของเชื้อรา *N. rileyi* เป็นเหตุให้สปอร์ของเชื้อราที่ได้จากอาหารเลี้ยงเชื้อสูตร FMAY นั้นยังคงคุณสมบัติในการงอกจึงทำให้สามารถงอกได้เร็วกว่า สปอร์ของเชื้อราที่ได้จากอาหารเลี้ยงเชื้อสูตร SMAY นอกจากนี้ยังได้ทำการศึกษาเปรียบเทียบรูปแบบของโปรตีนที่ถูกสร้างขึ้นในระหว่างการงอกของสปอร์โดยวิธี SDS-PAGE พบว่า virulent strain มีการสร้างโปรตีนที่แตกต่างจาก attenuated strain ซึ่งจากการวิเคราะห์ขนาดของโปรตีนที่แตกต่างกันเหล่านั้นพบว่ามีขนาดเท่ากับ 43 และ 25.04 kDa ในขณะที่โครงสร้างของโปรตีนดังกล่าวยังไม่สามารถระบุได้อย่างชัดเจน ส่วนผลการศึกษา บทบาทของ cuticle degrading enzymes ต่อความสามารถในการฆ่าหนอนพบว่าเอนไซม์ protease น่าจะมีส่วนช่วยในการแทงผ่านลำตัวของหนอน ในขณะที่เอนไซม์ chitinases และ lipases นั้นเชื่อว่าอาจจะมีความจำเป็นต่อการเจริญเติบโตของเชื้อราเท่านั้น

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LIST OF ABBRIVIATIONS

bc	blood cell
bp	base pair
BSA	Bovine serum albumin
C	conidium
°C	degree Celsius
cm	centimeter
Cp	conidiophore
cu	cuticle
D	dermis cell
ep	epicuticle
epd	epidermis cell
et al.	Et.alli (Latin), and others
etc.	ec cetera (Latin), and other things, and so on
ex	exocuticle
fig.	Figure
FMAY	fish soluble agar medium supplemented with 1- % yeast extract
g	gram
ge	gut epithelial cell
gl	gut lumen
GLC	Gas liquid chromatography

LIST OF ABBRIVIATIONS

(Continued)

GlcNAc	*N*-acetylglucosamine
Gt	germ tube
h	hour (s)
hb	hyphal body
he	Hemocoel
hp	hyphae
i.e.	Ed est (Latin), that is
kDa	kilodalton
L, l	litre
LC_{50}	the median lethal concentration produces death in 50% of the infected larvae within a certain time
LT_{50}	the median lethal time is the time required to produce death in 50% of the infected larvae to a specific dosage of pathogens
λ	wavelength
m	mili-/muscle
MeOH	methyl alcohol
mg	milligram
ml	milliltre
mM	millimolar
M	molar

LIST OF ABBRIVIATIONS

(Continued)

min	minute
MW	molecular weight
nm	nanometre
Na_2CO_3	Sodiumcarbonate
nu	nuclei
OD	optical density
PH	phialide
PCR	polymerase chain reaction
rpm	round per minute
SDS-PAGE	sodium dodecyl sulfate polyacrelamide gel electrophoresis
SMAY	Sabouraud's maltose agar medium supplemented with 1-% yeast extract
TCA	Trichloroacetic acid
TE	Tris-EDTA
TEMED	N-N-N'-N'-tetramethyl ethylenediamine
U	unit
UV	ultraviolet
V	voltage
v	volume
wt	weight

LIST OF ABBRIVIATIONS

(Continued)

μg	microgram
μl	microlitre
μmol	micromole
%	percent

CHAPTER I

INTRODUCTION

The tobacco cutworm, *Spodoptera litura*, which is commonly found throughout Thailand, belongs to the Lepidoptera. It is a polyphagous insect attacking many crops and is regarded as a serious pest in many parts of Thailand. Control of this insect, currently, mainly relies on applications of chemical insecticides. There are concerns on environmental problems related to the use of chemical insecticides. The consistent use of chemical insecticides is led to the development of resistant to those insecticides. Microbial insecticides using fungi are the alternative way to control insect pest. Several advantages of these biological agents, i.e., they are able to sustain in the environment, specific activity toward target host and reduce the probability of pest resistance. The first practical attempt using fungus as the biological control agent was conducted by Metschnikoff and reported in 1879. It is believed that there are more than 700 species of fungi have been recorded as insect pathogens. Although some of these have been investigated and commercially developed, the fungal products based on *Beauveria bassiana, Metarhizium anisopliae, Paecilomyces fumusoroseus, Verticillium lecanii* and *Nomuraea rileyi* are currently used to control insect pests in many countries.

The purpose of this study was aiming at studying factors contributed to the growth and pathogenicity of the local isolate of *Nomuraea rileyi* against *Spodoptera litura.* It has been shown that *Nomuraea rileyi* can be grown rapidly with strong virulence against caterpillar pests. This fungus can be successfully cultured in the large scale and it can be cultured *in vitro* at laboratory level. Many investigators have demonstrated that this organism could grow on an artificial diet, Sabouraud's maltose medium supplemented with 1 % yeast extract (SMAY). However, after several serial passages of conidia on artificial diets, it turned out that the growth, sporulation and virulence of the fungus were scanty. Efforts to avoid those aforementioned disadvantages were carried out.

CHAPTER II

LITERATURE REVIEW

1. Microbial Control of Insect Pest

Extensive use of synthetic chemical insecticides has resultrd in a variety of problems. Environmental concerns of hazards derived from chemical insecticides have led to increasing emphasis on the development of alternative ways by using biocontrol agents. However, they still play a minor role, despite considerable progress in the last decades, accounting for an estimated of 0.4 to 0.6% of the total pesticides market worldwide. The microbial pesticides currently marketing are dominated by *Bacillus thuringiensis* based products, whereas products derived from strains of viruses, i.e., nuclear polyhedrosis virus and granulosis virus, and from fungi are very little. Nevertheless, the potential of fungal entomopathogens as insect pest control agent is recently recognized. There are several fungal products currently commercial available, i.e., products from *Beauveria bassiana, Metarhizium anisopliae, Paecilomyces farinosus, Verticillium lecanii* and *Nomuraea rileyi* as summarized in Table1.

Table 1. Some common fungi that can parasitize insects and arthopods (1).

Fungus	**Hosts***	**Distribution**
Metarhizium anisopliae (Deuteromycota)	Many: Lepidotera, Coleoptera, Orthoptera, Heminoptera, Hymenoptera	Worldwide
Beauveria bassiana (Deuteromycota)	All	Worldwide
Hirsutella thompsonii (Deuteromycota)	Arachnida (mites)	Widespread
Cordyceps militaris (Ascomycota)	Many larvae and pupae of Lepidoptera, some Coleoptera and Hymenoptera	Worldwide
Nomuraea rileyi (Deuteromycota)	larvae and pupae of Lepidoptera, some Coleoptera	Worldwide
Paecilomyces farinosus (Deuteromycota)	Many: Lepidotera, Diptera, Homoptera, Coleoptera, Hymenoptera, Arachnida	Worldwide
Verticillium lecanii (Deuteromycota)	Several, especially aphids, scale insects	Widespread, tropics and sub-tropics
Entomopthora, Erynia and similar fungi (Zygomycota)	Various, often host-specific, e.g. *Entomophthora muscae* on housefly, *Erynia neoaphidis* on aphids	Worldwide
Coelomomyces species (Chitridiomycota)	Mosquitoes and midges; often host-specific	Common

*Lepidoptera-butterflies and moths; Diptera-flies; Homoptera-bugs; Coleoptera-beetles; Hymenoptera-wasps and bees; Orthoptera-grasshoppers, locusts; Heminoptera-sucking bugs; Arachnida-spiders and mites

2. History of *Nomuraea rileyi*

Farlow first described the entomopathogenic fungus, *N. rileyi*, as *Botrytis rileyi* in 1883 (2). In fact, the taxonomy was not clarified at that time. It was reclassified as a member of genus *Spicaria* and subsequently to genus *Paecilomyces*. Finally, It was placed in genus *Nomuraea* and the name was changed from *Spicaria rileyi* to *Nomuraea rileyi* (3). There are currently three described species in the genus *Nomuraea*. They are *Nomuraea rileyi, N. atypicola,* and *N. anemonoides*. The most widely known species, *N. rileyi,* was isolated from insects, primarily Lepidopteran larvae. The second species, *N. atypicola,* was isolated from spiders while the third species, *N. anemonoides,* was isolated from Australian soils. All three species of *Nomuraea* are taxonomically related but occupy different biological niches (4).

The first attempt using *N. rileyi* as biological control agent took place in 1955 by Chamberlin and Dutky (5). It was also commonly known that *N. rileyi* induced extensive epizootics in caterpillar pests on cabbage, clover, soybeans and velvet beans and thus was a potential candidate for use as a microbial insecticide.

3. Biology of *Nomuraea rileyi*

N. rileyi is a dimorphic hyphomycete having invasive hyphal stages and a yeast-liked vegetative stage. Initial growth begins with yeast-liked budding from germ tube of conidium. After a few days these yeast-liked hyphal bodies produce a cream colored, sticky growth and musty odor on agar surface. Sporulation is initially localized and then spread throughout the colony. The color of the colony progresses from white to pale green and subsequently to malachite green. Vegetative hyphae, 2-3 μm in diameter, are smooth, septate, and hyaline to slight pigmented. Conidiophores, which grow from submerged hyphae, are erect, septate, up to 160 μm in length and 2-5 μm in diameter. Branches, formed near a septum, develop in whorls each giving rise to 2-4 phialides. The branches, 5 - 8 x 2 - 4 μm, are usually cylindrical, occasionally with a swollen base. Conidia, in dry divergent chains, are smooth, ellipsoidal, pale green and 3.4 - 4.5 x 2 -3.1 μm (6). It was shown that conidia contributed to pathogenicity of the fungus toward insect host. The germination of conidia is optimal between 15 – 25 °C with high humidity. The growth of *N. rileyi* can occur on both solid and liquid substrate. Therefore, the study of conidia production and germination are normally achieved in solid substrate while the liquid broth allows for the production and characterization of extracellular hydrolytic enzymes. It has been postulated that the production of these enzymes may involve in its pathogenicity. The detection of cuticle-degrading enzyme expressed during germination of conidia and growth may be related to the difference between species and virulence of *N. rileyi.*

4. Mode of Action

The *in vivo* life cycle of *N. rileyi* has been reported for several host insects (7), (8), (9). The infection process of *N.rileyi* begins with an attachment of conidia to the insect integuments. It is believed that these conidia are produced cuticle-degrading enzymes, chitinase, protease and lipase, during this process (8). Once hyphae reaching the haemocoel, it will produce yeast-liked hyphal bodies where the fungus replicates by budding and septation in the haemolymph. Then the noninvasive hyphal bodies, which fill the host haemocoel, synchronously convert to an invasive mycelial stage. At the end of the infection cycle, hyphal bodies form elongating hyphae, which emerge from the cuticle and produce conidiophores. After insect death, the larval body is completely mummified and covered by dense white mycelium. The conidiophores produce a pile of pale green conidia about 1 - 2 days later.

5. Insects Host Range

The host spectrum of *N. rileyi* has been reported from several countries and summarized in table 2.

Table 2. Species of insects reported as being susceptible to *Nomuraea rileyi* (6)

Insect species	**Occurrence**	**Food plant**	**Location**
Coleoptera			
Hypera punctata	natural	clover	USA
Leptinotarsa decemlineata	laboratory	-	France
Lepidoptera			
Achoea janata	laboratory	caster bean	India
Agrotis ipsilon	Natural, laboratory	soybean	USA
Amathes badinodis	natural	-	USA
Anticarsia gemmatalis	Natural, laboratory	velvetbean, soybean	Brasil, USA, Puerto Rico
Bombyx mori	laboratory	-	Japan
Chrysodeixis eriosama	natural	cabbage	Ceylon
Cosmia nr. *exigua*	natural	-	Fiji
Feltia ducens, F. gladiaria	laboratory	-	USA
Glyphodes phyloalis	natural	mulberry	Japan
Heliothis armigera	natural	cotton	Africa
Heliothis zea	natural	alfafa, corn, cotton and soybean	USA
Heliothis virescens	natural, laboratory, insectary	cotton, soybean	USA
Heliothis zea-virescens	natural	many	USA

Insect species	**Occurrence**	**Food plant**	**Location**
Hyphantria cunea	natural	mulberry	Japan
Lymantria dispar	laboratory	-	USA
Mythimna (pseudaletia) unipuncta	natural	rice	USA
Ostrinia nubilalis	natural	corn	USA
Peridroma saucia	laboratory	soybean	USA
Pieris rapae	natural	cabbage	USA
Pionea forficalis	natural	-	-
Plathypena scabra	natural, laboratory	Soybean	USA
Pseudaplusia includens	natural, laboratory	cotton, soybean	USA
Spodoptera exigua	natural, laboratory	Soybean gram, millet	USA
Spodoptera frugiperda	natural	-	USA, India colombia
Spodoptera litoralis	natural	cotton	Israel, Madagascar
Spodoptera litura	natural	tobacco	India
Spodoptera ornithogalli	natural	-	USA
Stenachroia elongella	laboratory	jowar	India
Trichoplusia ni	natural, laboratory	cabbage soybean	USA, Taiwan

6. Loss in Activity of *Nomuraea rileyi*

It was shown that the loss of virulence in insect hosts of *N. rileyi* occurred after serially passed *in vitro* (10). It was proposed that this fungus altered its growth and development both on *in vitro* and *in vivo* substrate. It was found that only six passages of conidial transfer on Sabouraud's maltose and yeast extract agar plate resulted in a reduction in the ability of these culture to produce the yeast like, hyphal bodies stage. However, B.J. Morrow and C.M. Ignoffo reported that conidia derived from the 6th to 10th passages failed to produce conidia on infected larva even though they are virulent to neonatal larvae. Furthermore, conidia from the 16th passage were avirulent to larvae when applied topically to neonatal larvae of *Anticarsia gemmatalis*. An understanding of instability of *N. rileyi* and subsequent attenuation to target host insect is vital for its development to use as a successful microbial control agent.

7. Biology of *Spodoptera litura*

The tobacco cutworm, *Spodoptera litura*, which is commonly found throughout Thailand, is classified as a member of the Lepidoptera. It is a destructive pest of subtropical and tropical agriculture, and seems to be a serious pest in Thailand. The larval stages feed on a wide range of plants, including edible and ornamental crops.

7.1 Life cycle

Adult females of *Spodoptera litura* are normally laid their eggs in an irregular furry mass on the underside of a leaf. The caterpillars initially have a translucent green in color with a dark thorax. The young caterpillars are smooth-skinned with a pattern of red, yellow, and green lines, and with a dark patch on the mesothorax. They feed mainly on the flesh of the leaves leaving the veins intact. The later stages of larvae feed on whole leaves, flowers and fruits. They become brown with three thin yellow lines down the back, one in the middle and others on each side. A row of black dots runs along each side, and a conspicuous row of dark triangles decorates each side of the back. The last instar larva develops into a very dark color with four prominent yellow triangles on the mesothorax. The caterpillar curls into a tight spiral with the head protected in the center when they are disturbed. The caterpillar burrows into the soil below the plant several centimeters deep from the soil surface and pupates without a cocoon. The duration to become a pupal stage is around two weeks depending on the temperature. The adult moth is brown in color with a complex pattern of cream streaks across the fore wing. The hind wings are silvery white in color. It has a wingspan of about 4-cm long. The males have a blue-gray band from the apex to the inner margin of each fore wing.

7.2 The basic structure of insect cuticle

The cuticle is a secretion of the epidermis and covers the whole body. It is differentiated into two major regions: an inner region, up to 200 μm thick, characterized by the presence of chitin and forming the bulk of the cuticle, and the thin outer epicuticle, 1-4 μm thick, which contains no chitin (11).

7.2.1 Chitinous cuticle

Chitin is a characteristic constituent of insect procuticle commonly comprising 20-50% of its dry weight. The insect cuticle is always associated with protein, perhaps being bound to it by covalent bonds. Chitinous cuticle as it is first secreted is known as procuticle. Subsequently, the outer part of cuticle often becomes hard and rigid to form exocuticle while the inner, undifferentiated part is called endocuticle (Fig 2).

There may be a region between the two parts of hardened, but not fully darkened cuticle is called mesocuticle.

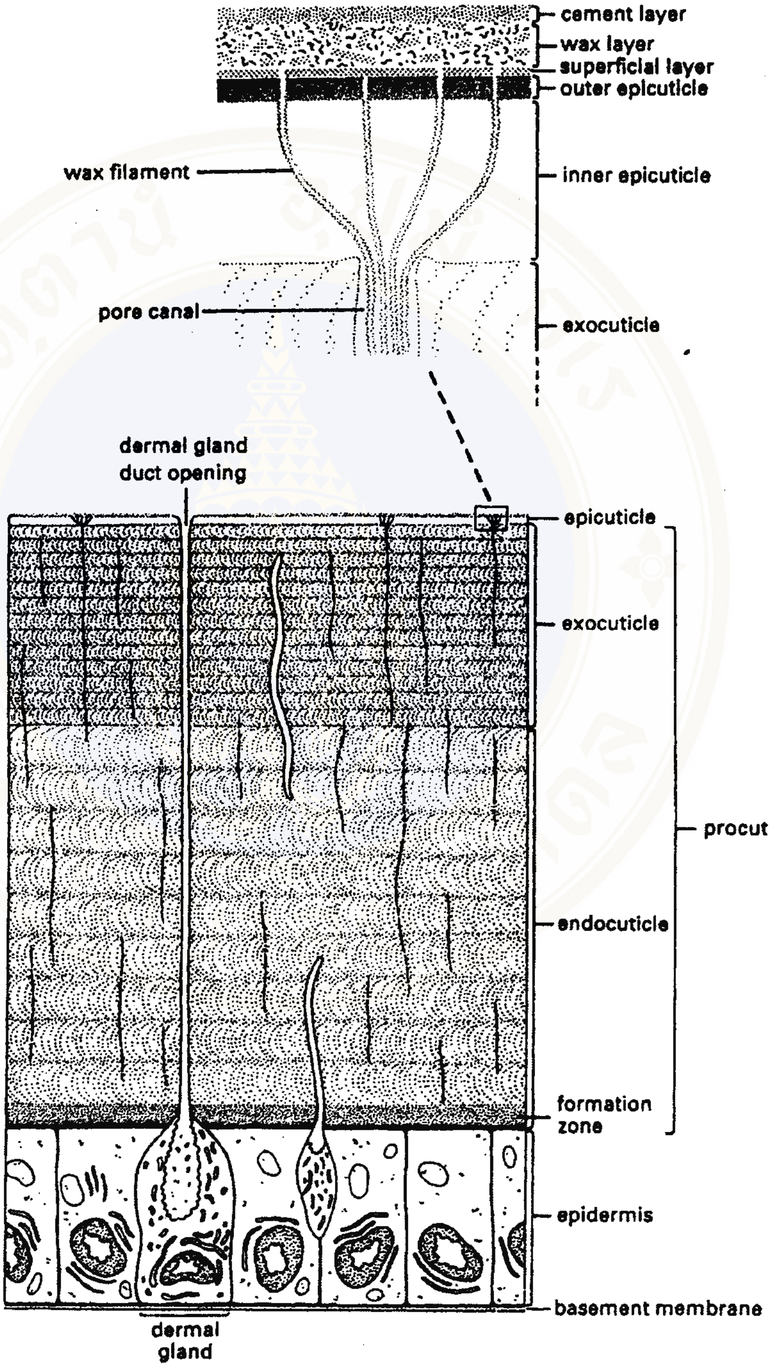

Figure 1. The general structure of insect cuticle showing details of the epicuticle (12).

7.2.1.1 Chitin

Chitin is a polysaccharide made up largely of *N*-acetylglucosamine residues, but it also probably contains some glucosamine. The sugar residues are linked by 1-4β-linkages so that they form a chain in which all residues are oriented in the same direction. Adjacent chitin chains are held together by hydrogen bonds to form microfibrils. Neighboring chains run in opposite ends of the residues.

The chitin microfibrils are about 2.5 - 3.0 nm in diameter and are embedded in a protein matrix. They lie parallel to each other in the plane of cuticle, but their orientation is often different in successive level through the thickness of the cuticle. In the most insects, the microfibrils in the outer parts of the procuticle, which subsequently become the exocuticle, rotate counter clockwise through a fixed angle in successive levels so that their arrangement is helicoidal and a series of thin lamellae is produced. This is called lamellate cuticle. The inner procuticle may also be lamellate throughout, or layer with helicoidally arranged microfibrils are uniformly oriented. The whole lamellate cuticle is found in Apterygota and in larval and pupal stages of Lepidoptera, Diptera and Coleoptera. The helicoidal and unidirectional layers alternate orientation and all the unidirectional layers may have the same orientation as those found in locust and cockroaches, or they may have different orientations as in beetles and bugs.

7.2.1.2 Protein

Proteins are the major constituents of insect cuticle. A hundred or more are present in the cuticles of most insects. Cuticle from any one part of an insect contains several different proteins, which may differ from the proteins in the cuticle of the other parts of the same insect. Proteins produced by one family of genes are characteristically associated with membranous regions of the cuticle, while proteins produced by another gene family characterized hard cuticle. The differences are apparent even before the cuticle is hardened, and the differing physical properties of different parts of the cuticle appear to be at least partly a consequence of the different proteins they contain, independent of the hardening process. The proteins of soft cuticle generally contain more aspartic acid, glutamic acid, histidine, lysine and tyrosine and are more hydrophilic than those of hard cuticles. The same proteins are present in cuticle with similar from different stages of an insect: the proteins appear to characterize the type of cuticle rather than the stage of development.

Hardening of cuticle is primarily a consequence of cross-links between protein molecules so that they form a rigid matrix. The process of cross-linking is called tanning or sclerotization and the cuticle is then said to be sclerotized.

7.2.1.3 Lipid

Lipids are present in the procuticle and, in *Rhodnius*, they impregnate the walls of the pore canals and are present in layers at intervals of 0.5-1.0 µm. These lipid layers become dispersed when the insect feeds and the cuticle stretches, but more layers are laid down with the new cuticle. Wigglesworth (1988) believed that lipid, together with the sclerotized proteins, plays a significant role in cuticle hardening.

7.2.2 Epicuticle

The epicuticle is made up of several layers. The thickest layer, 0.5 to 2.0 µm thick, is the inner epicuticle immediately outside the procuticle. Outside it, is a very thin outer epicuticle, only about 15 nm thick, and outside this again is a wax layer of variable thickness. Some insects have a thin “cement” layer outside the wax.

7.2.2.1 Inner epicuticle

The inner epicuticle layer is chemical complex and is known to consist primarily of tanned lipoproteins. Phenolic substances and phenoloxidase are also present during the production of this layer. These substances are probably concerned with tanning the proteins. Phenoloxidase persists as an extracellular enzyme in mature cuticle, producing further tanning if the epicuticle is damaged.

7.2.2.2 Outer epicuticle

The outer part of epicuticle is a very thin trilaminar layer. It is a highly polymerized lipid, and probably also has a protein component. Polyphenols and phenoloxidase take part in its formation. It is the first-formed layer of new cuticle produced at each molting period, protecting the new procuticle from the molting enzymes. It is believed to be inextensible, setting a limit on any extension of the procuticle during growth or other activities. The material forming the outer epicuticle is often referred to as cuticulin.

7.2.2.3 Wax

The epicuticular wax layer contains many different compounds as shown in figure 3. Hydrocarbons are universally present, and may comprise over 90 % of wax, as found in the cockroaches. Chain length ranging from 12 to over 50 carbon atoms, and compounds with an odd number of carbon atoms in the chain are usually dominant. Aliphatic alcohols are the most abundant compounds in larval Lepidoptera and Coleoptera. In this case, compounds with even numbers carbon atoms, in the range 12 to 34, are dominant. The alcohols may form esters with fatty acids, but esters are usually only minor components. In an adult stonefly, free fatty acids constitute the principal class of compounds in the wax.

The wax is important in waterproofing the cuticle and, in some insect, is the source of chemical signals important in intra-specific and, perhaps, interspecific signaling. It is synthesized by the oenocytes.

7.2.2.4 Cement

The cement is a very thin layer outside most of the wax, perhaps consisting of muco-polysaccharide, which become closely associated with lipids. It may serve to protect the underlying wax, although it is sometimes present as an open meshwork. It is not produced by all insects and appears to be absent from the cuticle of honeybees, for example. Cement is the product of type 3 gland cells in the epidermis

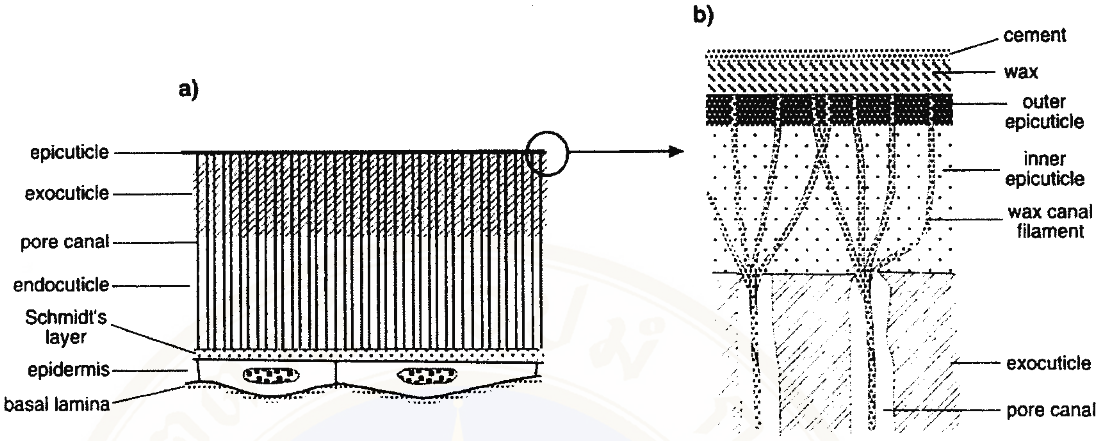

Figure 2. Basic structure of the insect integument (11).

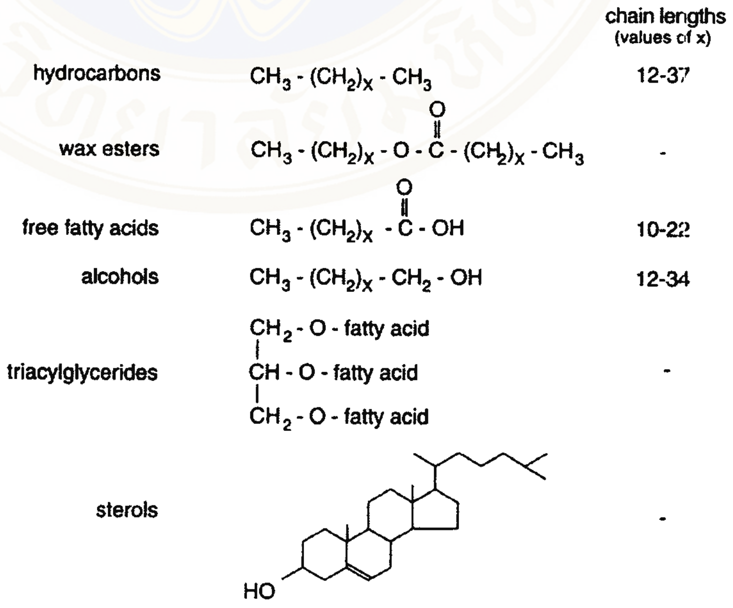

Figure 3. The structures of main groups of compounds occurring in epicuticular wax (11).

8. Toxicity Bioassay

Bioassay against insect larvae can be performed in many methods. The methods that commonly used are larvae force-feeding experiment; larvae feed on leaves of plants that have been coated with solutions to be assayed by dipping or spraying, larvae feed on artificial diet with incorporated materials or surface treatment, and larvae topically applied with the agent. The factors that influenced the choice of assay procedures are the type of insect used, growth rates, eating habits and their behaviors. Moreover, the choice of life stage and environmental conditions may all affect the results of the bioassay and thus must be carefully considered.

The toxicity is normally expressed in term of the median lethal concentration (LC_{50}) that produces death in 50% of the infected larvae within a certain time. The unit of LC_{50} value is spore/larva. The median lethal time (LT_{50}) is the time required to produce death in 50% of the infected larvae to a specific dosage of pathogens. The LC_{50} and LT_{50} values can be calculated by using a Probit analysis.

9. Cuticle-Degrading Enzymes of Entomopathogenic Fungi

The mode of entry for most entomopathogenic fungi is a direct penetration of host cuticle. Ultrastructural and histochemical studies suggest that the penetration occur via a combination enzymatic degradation and mechanical pressure (13).

The enzymatic degradation of host cuticle may be resulting from either extracellular enzymes or similar enzymes that remain bound on the cell wall of the pathogen. Extracellular enzymes corresponding to the main chemical constituent of insect cuticle, i.e., protein, chitin, and lipid, have been detected in various entomopathogenic fungi. However, information on types, characteristics, mode of action, regulation, cellular localization and rate, sequence, and levels of production are not fully known. Thus, the effects of cuticle-degrading enzymes to fungal penetration, the extracellular cuticle-degrading enzymes, chitinolytic, proteolytic and lipolytic enzymes were determined in various isolates of *N.rileyi* grown in liquid culture.

10.Chitinolytic Enzymes

Chitinase was first described in 1911 by Bernard. It plays important roles in the degradation of chitin, which is an insoluble linear β-1,4-linked polymer of N-acetylglucosamines (GlcNAc). Chitinases are defined as enzymes cleaving a bond between the C1 and C4 of two consecutive N-acetylglucosamines of chitin. Endochitinases, exochitinases (EC 3.2.1.14), β-N-acetylglucosaminidases and chitobiases (EC 3.2.1.30) have been characterized. Usually β-N-acetylglucosaminidases is defined as an enzyme releasing N-acetylglucosamine monomers from chitin, exochitinases as an enzyme releasing chitobiose and endochitinases as an enzyme splitting within the chitin polymer. Chitobiase hydrolyses chitobiose. Some chitinases also display a more or less pronounced lysozyme activity (EC 3.2.1.17) corresponding to the cleavage of a glycosidic bond between the C1 of N-actylglucosamine in the bacterial peptidoglycan. Transglycosidase activites associated with exochitinase activities have also been detected (14).

Chitinase has been found in various microorganisms such as bacteria, fungi, plant and invertebrates. The physiological functions of chitinase are depended on their sources. Bacteria produced chitinase to digest chitin and utilized it as carbon and energy source. Protozoa produced chitinases in order to complete its life cycle. Plant chitinase is thought to be a defense mechanism in response to pathogens. Fungal chitinases seem to play a physiological role in cell division and differentiation. Moreover, Chitinase could also be involved in the penetration of a host by fungi.

10.1 Fungal chitinases

All type of chitin-degrading activities were found in fungi. Endochitinases and β-N-acetylglucosaminidase was found in *Apergillus nidulans* (15). Exochitinases was found in *Mucor rouxii* (16). Nine chitinases species were detected in germinating cells as well (17).

11.Proteolytic Enzymes

The term of protease refers to all enzymes that hydrolyze peptide bonds. Other names include peptidase and peptide hydrolase are also used. These enzymes can be subdivided into exopeptidases and endopeptidase based on their activities. Endopeptidase is used synonymously with proteinase (18).

Proteases are classified into four groups according to the catalytic residue involved in the nucleophilic attack at the carbonyl carbon of the scissile bond. They are serine (EC 3.4.21), cysteine (EC 3.4.22), aspartic (EC 3.4.23), and metalloproteinases (EC 3.2.24), respectively. Proteinases from mold can be classified as acidic, neutral, or alkaline on the basis of the pH dependence of enzyme activity as summarized in table 3.

The physiological functions of protease in entomopathogenic fungi are normally involved with the degradation of the proteinaceous part of outer integument of insects. There is the evidence that the ability of fungi to degrade protein may aid their invasion and growth in this complex structure. Evidence for the particular importance of proteinases derives largely from the studies of their production in infected cuticles associated with cuticle degradation, the effects of proteinase inhibitors on pathogen behavior, and by the analysis of protease-deficient mutants. More recently, studies have included the cloning, identification, and manipulation of specific protease gene of *Metarhizium anisopliae*, such as subtilisin-like proteinases (Pr1), metalloproteinase (M.pr.), trysin-like proteinases (Pr2), carboxypeptidase, aminopeptidases, and dipeptidyl (dip) peptidase. It has been shown that Pr1 also produced by many other entomopathogenic fungi (19). Utilization of these enzymes has assisted investigators in understanding cuticle structure and how the cuticle is degraded naturally, and could lead to improved strain selection of entomopathogenic fungi or the introduction of their genes into other microbes and plants for the propose of insect control.

Table 3. Properties of protease from molds (20).

Organism	pH range for		Optimal pH for stability	Inhibitors*
	Hemoglobin	Casein		
Aspergillus saitoi	3.0-4.5	2.5-3.0	2.0-5.0	NBS
Aspergillus oryzae (acidic protease)	3.0-4.0	2.5-3.0	5.0	
Aspergillus oryzae (neutral protease)	5.5-7.5		7.0	EDTA
Aspergillus oryzae (alkaline protease)	6.0-9.5	6.5-10.0	7.0-8.0	DIFP
Paecilomyces varioti	3.5-5.5	3.0	3.0-5.0	
Rhizopus chinensis	5.0	2.9-3.3	3.8-6.5	
Mucor pusillus	3.5-4.5	5.6	3.0-6.0	

- NBS=N-bromosuccinimide; EDTA= ethylenediaminetetraacetic acid; DIFP=diisopropylfluorophosphate.

12.Lipolytic enzymes

Lipolytic enzymes are composed of two major groups, the lipases, which are triacylglycerol acylhydrolase (EC 3.1.1.3) and the phospholipases A_1 (EC 3.1.1.32) and A_2 (EC 3.1.1.4), which are phosphoglyceride acyl hydrolases. Although phospholipases C (EC 3.1.4.3) and D (EC 3.1.4.4) are not acylhydrolases, they are nonetheless commonly included as lipolytic enzymes. The triacylglycerol lipases are found widely in animals, plant and microorganisms. Animal lipases include pancreatic, gastric, and intestinal lipases, and also lipases found in milk. However, the specificity of lipases varies considerably, and depends on the substrate chain length and on certain positions in the substrate molecule. Lipases can be isolated on a large scale from variety of sources (table 4).

Table 4. Comparison of properties of various lipase preparation (20).

Source (species)	pH range (pH optimum)	Temperature, °C	pH for stability
Porcine pancreas	6.5-9.5 (7.5-8.5)	40-45	5.5-7.5
Rhizopus species	6.0-7.5 (7.0)	35-40	4.0-8.0
Mucor juvanicus	5.5-8.0 (7.0)	40-45	4.5-6.5
Apergillus niger	a) 3.0-7.0* b) 7.5-9.0	40-50	5.0-7.0
Pseudomonas	a) 4.0-5.0**b) 7.0-8.5	50-60	4.5-10.0
Candida cylindracea	5.0-7.5	40-45	4.5-8.5

* pH 3.0-7.0 with short-chain fatty acids; pH 7.5-9.0 with fat and oils.

** Two pH optima for two isoenzymes.

CHAPTER III

MATERIALS AND METHODS

1. Isolation of the Fungus

Isolation attempts of local strains of entomopathogenic fungi were made from field collected diseased larvae of insect pest. The dead larvae of insect pest were collected from the northern part of Thailand, Amphoe Mae Chaem, Chiang Mai province during December 2000. Since the best characteristics of Mae Chaem strain are rapid growth and strong virulence, hence this strain was selected for this study. The specimens derived from mycelial mat with conidia of the fungus arising from different parts of the body of dead insect larvae were grown on artificial media in Petri dishes. The specimen was streaked across the surface of the medium. The artificial medium containing either fish soluble agar medium supplemented with 1% yeast extract (FMAY) or Sabouraud's maltose agar medium supplemented with 1% yeast extract (SMAY) was used. Chloramphenicol at the concentration of 25 μg/ml was added to both media for the suppression of bacterial contamination. The plates were incubated in a growth chamber regulated at 25 °C with 95 % relative humidity. The appearance of fungal colonies that grown on the surface of the artificial media was observed daily under the dissecting microscope. Only suspected green colony was separated and subcultured onto new fresh medium for the purification and identification.

To ensure that different characteristics observed in this experiment belong to the same fungus, the single-spore culture was performed for purification of this fungus. The conidia were serially diluted with 0.02% tween 80 in sterile distilled water and count the number of conidia under the light microscope, until not more than three conidia were found in a drop. Several drops were smeared onto an agar layer in a Petri dish. Those colonies grown on the agar surface were subsequently transferred onto fresh medium. This purified culture was maintained by infecting fresh larvae and subcultured onto artificial media in order to maintain its virulence for future use.

The purified culture of the fungal isolate was called as the 1st passage. The subsequent passage was obtained by transferring the conidia onto fresh medium, purified and maintained in artificial media.

2. Morphological Examination of the Isolate

The slide culture technique was use for the identification of the fungal isolate. A small block of agar was placed in the center of a sterile slide and each of the four edges was inoculated with the fungus. A sterile cover glass was placed on the top of the block. The slide was placed in a sterile Petri dish with a piece of filter paper (Whatman No.1) soaked with distilled water and kept in a moist chamber. The slide was incubated at 25 °C with 95 % relative humidity. The specimen was stained with Lactophenol-cotton blue and examined under the light microscope daily. Since it has been shown that *N. rileyi* also has a yeast-like vegetative stage with a cream colored and sticky growth colony. Thus, wet-mount technique was also used to examine the fungus.

3. Source of Insect Larvae and their Multiplication

A colony of *Spodoptera litura* was raised from field collected larvae and maintained in laboratory conditions. Adult females were allowed to lay eggs on cabbage leaves. Eggs were left on the surface of cabbage leaves until the 1st instar larvae were hatching out. The larvae were maintained in the cages until they reached the pupal stage. Then, the pupae were selected and used as a stock to build up new colonies. The adult insects were maintained in the cage supplemented with 10 % sugar solution.

4. Standard Strain of *Nomuraea rileyi*

An attenuated strain of *N. rileyi* was used as the standard strain for the comparison in characterization of the local isolates. It was obtained from Professor Dr. Drion G. Boucias Department of Entomology, University of Florida USA: strain No. 5758, 5762 and 5765.

5. Histopathological Studies

The late 2nd instar larvae of *S. litura* were used in this experiment. These larvae were topically applied with 5 μl of conidial suspension. The fungal suspension was prepared by diluting the stock of conidia with 0.02% tween 80 in sterile distilled water plus 0.05% stick agent. The final concentration used was 10^8 conidia/ml. The test insect larvae were placed in the growth chamber regulated at 25 °C with 95 % relative humidity after infection. The test larvae were observed at every 12-h interval until they were overgrowth with white mycelia. These larvae were fixed in Bouin's fluid for 24 h. Subsequently, they were washed in several changes of 70 % ethanol in order to remove the fixative agent. Dehydration was done through a series of graded ethanol for 15 min each. Then, they were embedded in paraffin wax. The paraffin that embedded with specimen was cut into a thin section at 5-6 μm thickness. Subsequently, sections were deparaffinized and stained with Harris's Haematoxylin and Eosin dye, mounted, and examined under the light microscope. Haematoxylin for nuclei and eosin as a general background stain.

6. Toxicity Bioassay

The late second instar larvae of *S. litura* were used throughout this experiment. The culture of *N. rileyi* of the given passage that was serially grown either on FMAY or SMAY medium was inoculated onto twenty larvae for each treatment. The attenuated strains received from Professor Dr. Drion G. Boucias was used as the standard control in parallel. The test cultures were prepared by harvesting and then diluted with 0.02% tween 80 in sterile distilled water plus 0.05% stick agent until they reach the following concentrations 10^8, 10^7, 10^6, 10^5, 10^4, and 10^3 conidia/ml, respectively. Since the later passages of *N. rileyi* culture on SMAY medium produced small amount of conidia, cultures on several containers were pool together before making the appropriate concentration. The 5 μl of conidial suspension was topically applied onto insect larvae. The test larvae were fed on cabbage leaves and placed in a growth chamber at 25°C with 95% relative humidity after infection. The mortality was assessed daily for 10 days and fresh diet was added as needed. The amount of dead larvae was recorded. The toxicity was expressed in terms of LC_{50} (spore per larvae) and LT_{50} (days). The LC_{50} and LT_{50} values were calculated by probit regression using the software; SPSS for windows Release 7.5 standard version, copyright© SPSS Inc., 1989-1996. The LC_{50} values were transformed to natural log and calculated according to maximum likelihood estimation and the chi-square goodness of fit was used to verify the fitness of the log probit model. The diluent composed of 0.02% tween 80 in sterile distilled water plus 0.05% stick agent was used instead of conidial suspension in the control group.

7. Investigation on Factors Attributing to the Virulence of *N. rileyi*

7.1 Measurement of Growth rate

The conidial cultures of the fungal isolate that grown on both media of 3rd, 6th, 9th, 12th, and 15th passages were used. All cultures except the 12th passage that grown on SMAY medium were suspended in 0.02% tween 80 in sterile distilled water, while cultures of the 12th passage were pooled together before making a suspension. The final concentrations of the suspension used were 1×10^8 spores/ml. The suspensions were inoculated into Sabouraud's maltose broth supplemented with 1-% yeast extract and 25 μg/ml of Chloramphenicol. The cultures were incubated at 25°C on a rotary shaker set at 150 rpm. Samples were taken at every 3 days interval until 12th day. The supernatants were harvested and clarified by filtration through the filter paper (Whatman No.1). The resultant pellets were dried by hot air oven at 60°C and used to determine their weight. The growth rate was determined and expressed as dried weight in g/min from the graph. The growth curve was done by plotting between the dried weight of fungal pellet and time course.

7.2 Determination of the Conidial germination

Germination attempts of conidia were carried out by spreading the 50 μl of conidial suspension at the concentration of 10^7 spores/ml on filtered FMAY plates and SMAY plates. The plates were incubated at 25°C and randomly used for preparing specimens to determine the germ tubes. The incubation times used in this experiment were at 24, 36, and 48 h, respectively. Several pieces of agar were randomly cut from the culture plate. A total of 300 conidia were examined microscopically for the presence of germ tube that appeared at least as long as the width of conidia.

7.3 Determination of fungal spore production in artificial medium

The experiment was run in parallel with the conidial germination experiment. The conidial suspension at the concentration of $1\text{x}10^8$ spores/ml was used and 100 μl of the suspension was inoculated into FMAY and SMAY slants. The slant culture was placed in the incubator at 25°C. The conidia were harvested at every 3 days interval and washed with mixture of 0.02% tween 80 that prepared in sterile distilled water. The amount of conidia was determined under the microscope with an aid of the hemacytometer (Hausser Scientific, U.S.A.).

8. Determination of Fish Soluble Components

The fat compositions of fish soluble medium were analyzed and identified by Food and Nutrition technical services, Institute of Nutrition, Mahidol University (INMU). The crude fat and fatty acid profiles were obtained by Soxhlet and Gas liquid chromatography (GLC) methods, respectively. The protein components of fish soluble were obtained by product specification from T.C. Union Agrotech Co., LTD.

9. Preparation of Fungal Supernatant for Enzyme Analysis

The standard strain No.5765 of *N. rileyi* and the culture of the 12th passage grown on SMAY medium were employed as attenuated strains. While the culture of the 2nd passage grown on FMAY medium was used as virulent strain in this study. The conidia were harvested from sporulated slant cultures and suspended in 0.02% tween 80 solution to make the final concentration at $1\text{x}10^8$ spores/ml. A two-ml suspension was inoculated into 200 ml of Sabouraud's maltose broth supplemented with 1% yeast extract and 25 µg/ml of Chloramphenicol. The cultures were incubated at 25°C on a rotary shaker set at 150 rpm. The supernatants were harvested from the cultures on 5th and 15th days and filtered by Whatman No.1 filter paper. The filtrates were centrifugation at 10,000 rpm for 30 min. The supernatant was concentrated by precipitation with 80% ammonium sulfate and used for SDS-PAGE and enzyme assay.

10.Ammonium Sulfate Precipitation (Salting Out)

Ammonium sulfate precipitation used in this study was based on a procedure described by Scopes, 1981. The supernatants were fractionated with solid ammonium sulfate on ice. Fine powder of ammonium sulfate was gradually added until the final concentration reached 80 % saturation. The mixtures were mixed continuously using a stirrer for 30 min followed by centrifugation at 10,000 rpm for 30 min at 4°C. The resultant pellets were resuspended in Sodium phosphate buffer, pH 7. Finally, the mixtures were subjected to remove ammonium sulfate by dialysis (Dialysis bag 5,000 daltons).

11.Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE used in this study was based on the procedure described by Laemmli, 1970.

11.1 Gel preparation

The SDS-PAGE was carried out with 12% separating gel using Biorad protein II. Preparations of separating gel and stacking gel are shown in Table 3. The separating gel was added about 1.5 cm from top of front plate. Then the gel solution was overlaid with distilled water and allowed to polymerized at room temperature for 30 min. Water was devoid from the gel prior to pouring the stacking gel on top of the separating gel and the well comb was inserted between the 2 plates. The gels were allowed to polymerized at room temperature for 30 min.

11.2 Sample loading and electrophoresis

The comb was carefully removed from the polymerized gel and the wells were cleaned by flushing several times with electrophoresis buffer (0.25 M Tris-HCl containing 0.192 M glycine, pH 8.3 and 0.1% SDS) to remove gel debris. The samples were prepared by mixing of 40 μl of crude protein with 10 μl of the 5x sample buffer (0.5 M Tris- HCl pH 6.8, 10% glycerol, 2% w/v SDS, 1% 2-b-mercaptoethanol, 0.025% bromophenol blue). After heating in boiling water for 5 min, 8.42 μg of protein samples were loaded onto the gel. The molecular weight markers were loaded in parallel. The two gels were electrophoresed in parallel at 150 volts for about 1 hour or until the tracing dye reached the bottom of the gel.

11.3 Coomassie blue staining

The gels were stained with Coomassie brilliant blue R-250 staining solution for 1-2 h in order to analyze the protein bands. The staining solution was consisting of 0.1% Coomassie brilliant blue R-250 in fixative solution (40% MeOH, 10% acetic acid). The gels were de-stained by soaking in de-staining solution (40% MeOH, 10% acetic acid) for 1-3 h. The stained gel were air dried in double layers of cellophane.

12.Determination of Protein Concentration in Crude extract

The concentrations of proteins were determined by a procedure described by Bradford, 1976. The Bradford assay was a rapid and reliable dye-based assay for determining protein content in a solution. The samples were determined by Bradford assay using bovine serum albumin (BSA) as a standard protein at the different concentrations of 0, 0.2, 0.4, 0.6, 0.8 and 1.0 mg/ml. The reaction mixture was consisting of 1 ml of dry reagent, the pierce Coomassie® Plus Protein Assay Reagent Kit, and 20 μl of sample. The mixture was allowed to stand at room temperature for 5 min, then the color was determined at wavelength 595 nm by the spectrophotometer (Phamacia Biotech, Novaspec® II). The absorbance was read against a blank preparation with the same buffer instead of the sample. The concentration of protein was determined by comparing with standard curve of bovine serum albumin (BSA).

13.Enzyme Activity Assay

13.1 Determination of Chitinase enzyme by colorimetric method

The activity of Chitinase enzyme was estimated by the colorimetric method described by Ueda and Arai (21) using colloidal chitin, glycol chitin as substrate and N-acetylglucosamine (GlcNAc) as the standard reducing group.

The reduction mixture was consisting of 0.2 ml of 0.1 M Mcllvaine buffer pH 7.0, 0.1 ml of 0.3% colloidal chitin (or 0.3% glycol chitin) and 0.1 ml of crude protein sample. The mixture was incubated at 37°C for 3 h with constant shaking at 200 rpm. One ml of potassium ferric cyanide solution was added as the coloring agent and subsequently heated for 15 min in boiling water. The potassium ferric cyanide solution was consisting of 0.5 g of potassium ferric cyanide in 1 L of 0.5 M Na_2Co_3. The negative control sample was consisting of all reagents except substrate and blank contained all reagents except enzyme. The release of N-acetylglucosamine in the reaction mixture was estimated by the spectrophotometer at the wavelength of 420 nm. The activity of chitinase enzyme was estimated from the standard curve of N-acetylglucosamine concentrations. One unit of the enzyme activity was defined as the amount of the enzyme able to liberate 1 µmol of N-acetylglucosamine per min under these standard assay conditions. Specific activity was expressed as unit per milligram of protein.

13.2 Determination of Chitinase activity by diffusion method

The lytic activity of chitinase enzyme was estimated by a method modified from the Diffusion method described by Tanaka and Phaff, 1905.

Ten ml of 1.5 % Bacto agar was poured into petridish as agar base. Then, the 10 ml of 1% Bacto agar supplemented with 0.3% colloidal chitin, pH 7.0 was overlaid on this agar base. The agar was allowed to solidified at room temperature. Cutting made a 9-mm diameter well with a cork borer, and 60 µl of crude protein was filled in the well. The plates were incubated at 37°C for 24 h or until the clear zone around the well had been seen, then the glycol chitin agar plate was overlaid with the brightening solution, the calcoflour white M2R (Sigma Chemical Co., St. Louis, MO). The clear zone of substrate hydrolysis was observed by the UV-transilluminator.

13.3 Determination of Protease enzyme by colorimetric method

Protease activity was estimated by the a modification of the colorimetric method according to Anson (22).

One ml of 1.5% Casein in 0.05 M phosphate buffer pH 7 was mixed with 1 ml of appropriately diluted crude enzyme with 0.05 M phosphate buffer pH 7. The mixture was incubated at 37°C for 1 h, following the reaction was stopped by 2 ml of 0.4 M Trichloroacetic acid (TCA). The mixture was allowed to deposit for 30 min and clarified by centrifugation at 10,000 rpm for 10 min. 0.5 ml of collected supernatant was added with 2.5 ml of 0.4 M Na_2CO_3 and 0.5 ml of 1 N folin-ciocalteu's phenol reagent. The developing color of reaction mixture after left standing for 10 min at room temperature was determined by spectrophotometer at the wavelength of 660 nm. The control of this assay was 1.5% Casein and crude enzyme that the reaction was instantly stop by 2 ml of 0.4 M Trichloroacetic acid (TCA). All reagents except crude enzyme was used as blank in this experiment. The protease activity was determined from the standard curve of Tyrosine concentrations. One unit of the protease activity was defined as the amount of the enzyme able to liberate 1 μmol of Tyrosine per min under these standard assay conditions. Specific activity was expressed as unit per milligram of protein.

13.4 Determination of Protease activity by diffusion method

One percentage of Bacto agar supplemented with 1 % skim milk was poured into petridish. After that, the agar was allowed to solidified at room temperature. Cutting made a 9-mm diameter well with a cork borer, and 60 μl of crude protein was filled in the well. The plates were incubated at 37°C after 24 h, the clear zone around the well was examined.

13.5 Determination of Lipase enzyme by colorimetric method

Lipase activity was estimated by the colorimetric method by the method according to Miles *et al.* (23). The mixture consists of the solution A (0.062 g of *p*-nitrophenylpalmitate in 10 ml isopropanol) and the solution B (0.4 v/v Triton X-100 and 0.1% w/v gum arabic in 50 mM Tris -HCl, pH 8.0) in 1:9 ratio. 0.2 ml of enzyme sample was added in 1.8 ml of the mixture. Then the mixture incubated at 37°C for 30 min. The release of *p*-nitrophenyl in the reaction mixture was estimated by the spectrophotometer at the wavelength of 410 nm. The lipase activity was determined from the standard curve of *p*-nitrophenyl concentrations. One unit of the lipase activity was defined as the amount of the enzyme able to liberate 1 μmol of *p*-

nitrophenyl per min under these standard assay conditions. Specific activity was expressed as unit per milligram of protein.

13.6 Determination of Lipase activity by diffusion method

One percentage of Bacto agar supplemented with 1% tributyrin (1,2,3-Tributyrylglycerol; C4:0) was poured into petridish. After the agar was allowed to solidified at room temperature. Cutting made a 9-mm diameter well with a cork borer, and 60 μl of crude protein was filled in the well. The plates were incubated at 37°C after 24 h, the clear zone around the well was examined.

CHAPTER IV

RESULTS

1. Isolation of the Fungus

A strain of *N. rileyi* was isolated from collected diseased larvae of *S. litura* in Amphoe Mae chaem, Chiang Mai province, Thailand. The fungus was isolated on the artificial media containing either fish soluble agar medium supplemented with 1% yeast extract (FMAY) or Sabouraud's maltose agar medium supplemented with 1% yeast extract (SMAY). The organism produced conidia with typical characteristics of *Nomuraea* species. It has yeast-liked hyphal bodies, a cream colored, sticky growth and musty odor on the agar. Sporulation was initially localized and then spread throughout the colony. The color of the colony was gradually changed from white to pale green to malachite green within 8 to 9 days. The yellow colony was occasionally observed.

2. Morphological Examination of *N. rileyi in vitro* Culture

Since the basic criteria used in the identification and classification of fungi are based on the characteristics of the spore, the slide cultures of *N. rileyi* were performed to determine the morphology, shape, size, and cell content of the fungal isolate. The isolate has a yeast-liked vegetative stage, a cream colored, sticky growth colony. It was examined under the light microscope using wet-mount technique.

The conidiophore of the isolate is erect and septate. It bears two or three compact phialides around the stalk. These phialides are usually short cylindrical and occasionally found with a swollen base, without neck or with a very short neck. The conidia are broadly ellipsoidal in shape and smooth walls, which produced at the phialides apex. The characteristics of the isolate are shown in figure 5.

The conidial germ tubes protruded from their polar ends after inoculation for 20 to 24 h as shown in figure 6. Subsequently, hyphal bodies started fragmenting themselves from germ tubes. The appearance of fragmented hyphal bodies became cream color and sticky colony after continuing to replicate on FMAY agar plate. The fragmentation of the hyphae usually occurred either at the apex of the germ tube or along hypha. The characteristics of hyphal bodies are shown in figure 7.

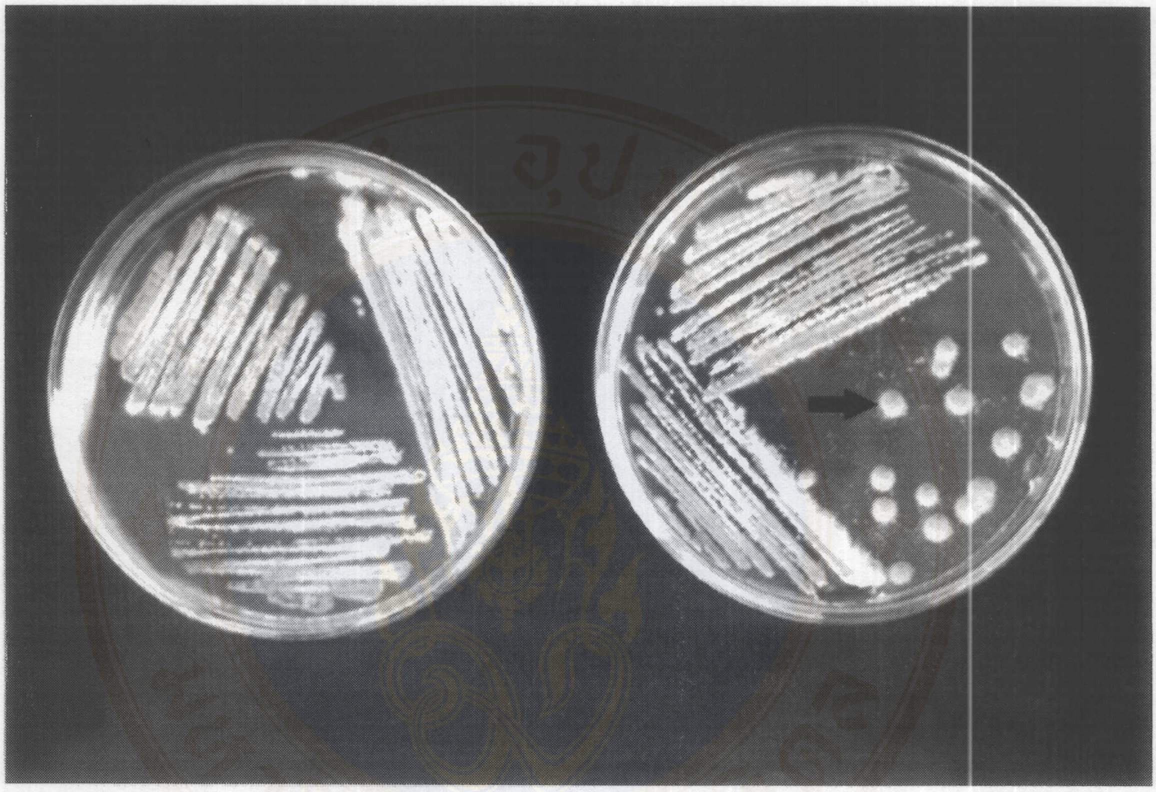

Figure 4. A picture of the 1st passage of the fungal isolate, *N. rileyi*, cultured on SMAY at 25°C for 10 days. Arrow shows the green conidia colony.

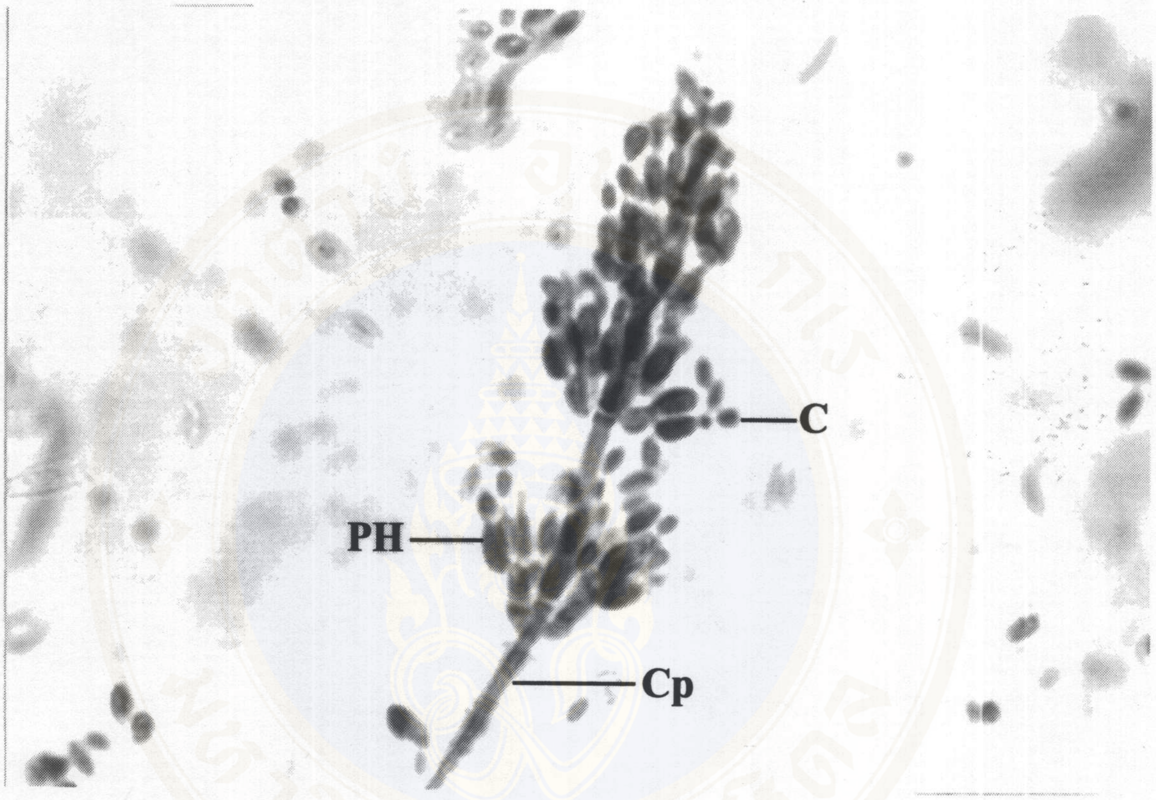

Figure 5. The formation of conidia of the fungal isolate, under light microscope at the magnification of 1000x.

Cp = Conidiophore

C = Conidium

PH = Phialide

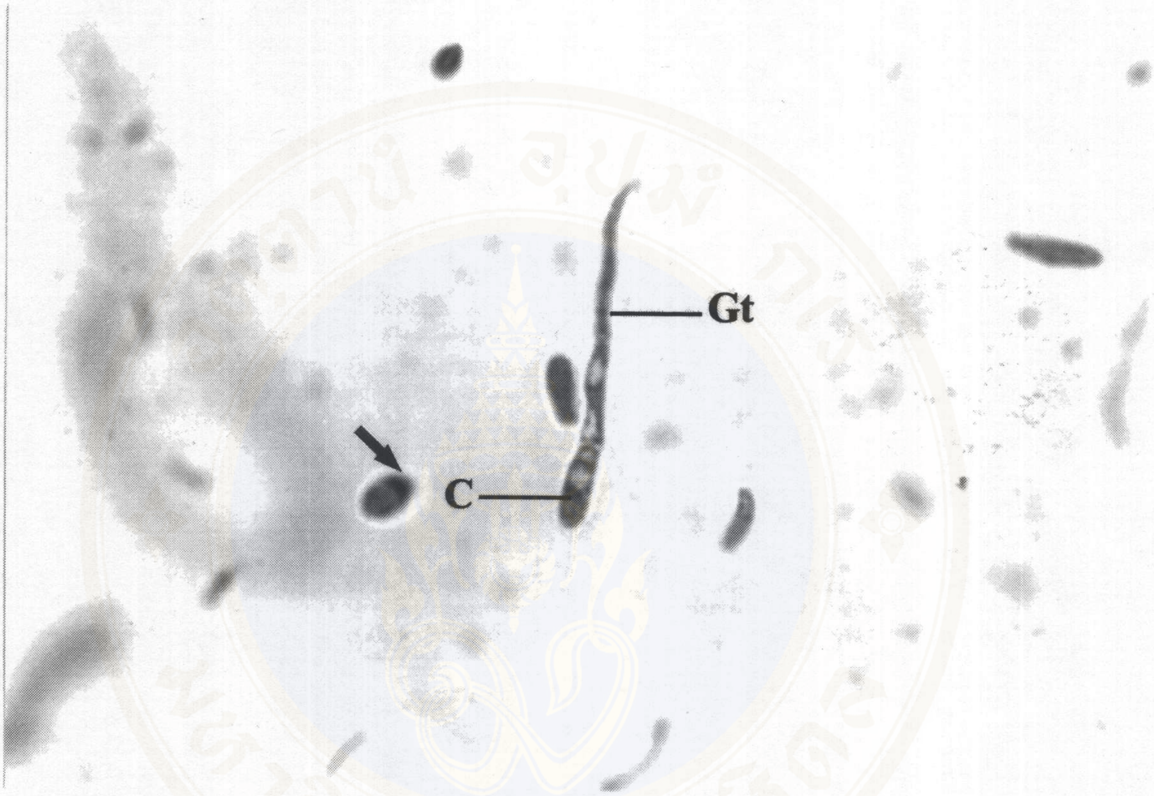

Figure 6. The formation of germ tube of the fungal isolate, *N. rileyi*, revealed under the light microscope at magnification 1000x. The conidial germ tube protruded from the polar end. Arrow indicates the polar end of conidia.

Gt = Germ tube

C = Conidium

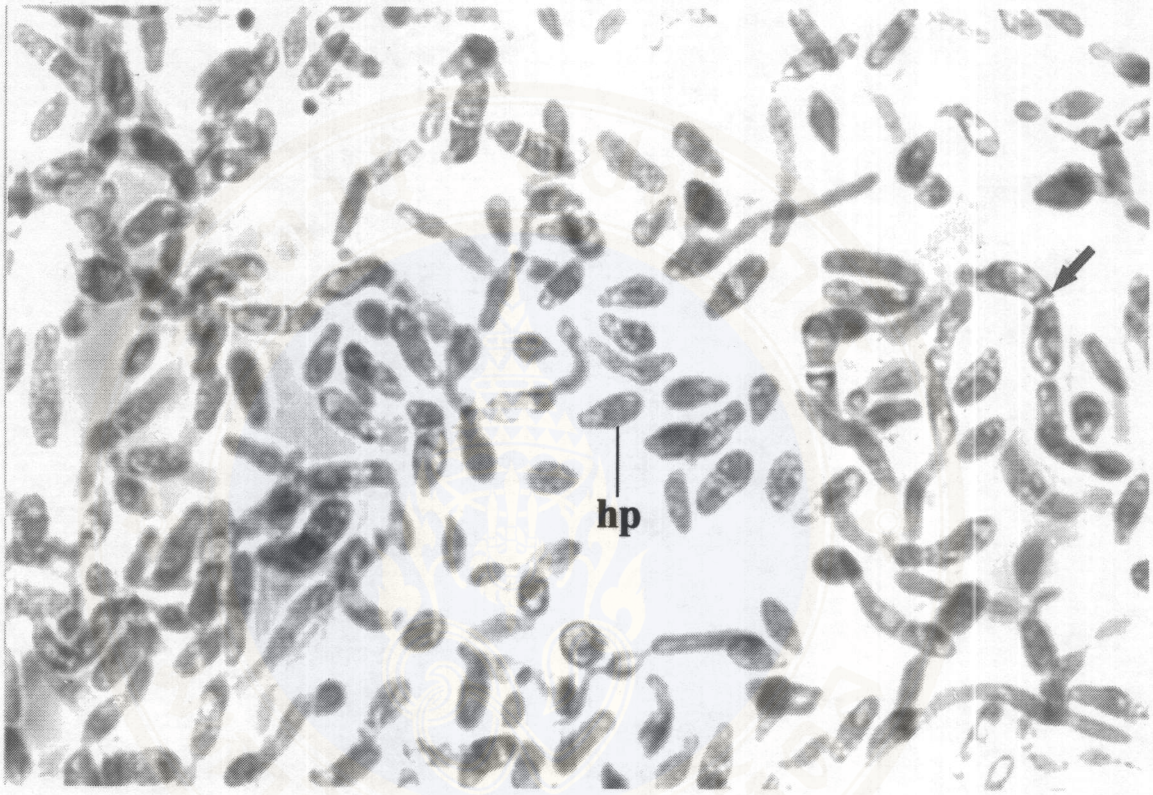

Figure 7. The formation of yeast-liked hyphal bodies of the fungal isolate, *N. rileyi*, found in a cream colored, sticky growth colony revealed under the light microscope at magnification 1000x. Arrow shows fragmentation and multiplication of hyphal bodies on FMAY

hp = Hyphal bodies

3. Histopathological Studies

The development of the fungal isolate was determined through sections of infected larvae under the light microscope. Sections made from non-infected larvae were used as control group and selected specimens are shown in figure 8A-B. The penetration of germ tubes through insect cuticle was observed within 48 h after inoculation without an appressorium formation. It was found that the hypha, developing germ tubes, grew along the endocuticle of insect larvae as shown in figure 9A. These laterally branched hyphae propagated between the epidermis and the exocuticle laminae of insect larvae. The lysis of endocuticle was also found before the invasion of hyphae to epidermis as shown in figure 9B. It was shown that hyphae had penetrated the epidermis and reached the haemocoel of insect larva 2.5 days after inoculation as shown in figure 10. Aggregations of blood cell were not observed at the points of entry to the haemocoel. The invasive hyphal stages switched to hyphal bodies stages and started replicating by budding and septating in the hemolymph as shown in figure 11. Noninvasive hyphal bodies filled up the haemocoel and cohabited with hemocytes. All tissues of infected larvae of *S. litura* were then colonized by the fungal isolate, *N. rileyi*, as shown in figure 13. Nevertheless, these processes had neither deleterious effect on feeding nor digestion of insect larvae and they seem to be healthy. Noninvasive hyphal bodies converted to invasive mycelia at 5.5 to 6 days after inoculation. These mycelia had completely ramified throughout all larval tissues and had begun to emerge through the cuticle as shown in figure 15. However, the emerging filamentous hyphae differed from that of laterally hyphae found in the early stage of infection as shown in figure 9A. The pile of mycelia made the dead larvae a completely white appearance. The larvae usually died with the anterior portion of its body in an elevated position. Newly formed conidia were visible on conidiophores at 7 days after inoculation. Subsequently, cadavers became completely covered by green conidia. These conidia rendered the cadavers appear green. The complete development cycle of the fungal isolate, *N. rileyi*, in *S. litura* larvae lasted approximately 8 to 9 days.

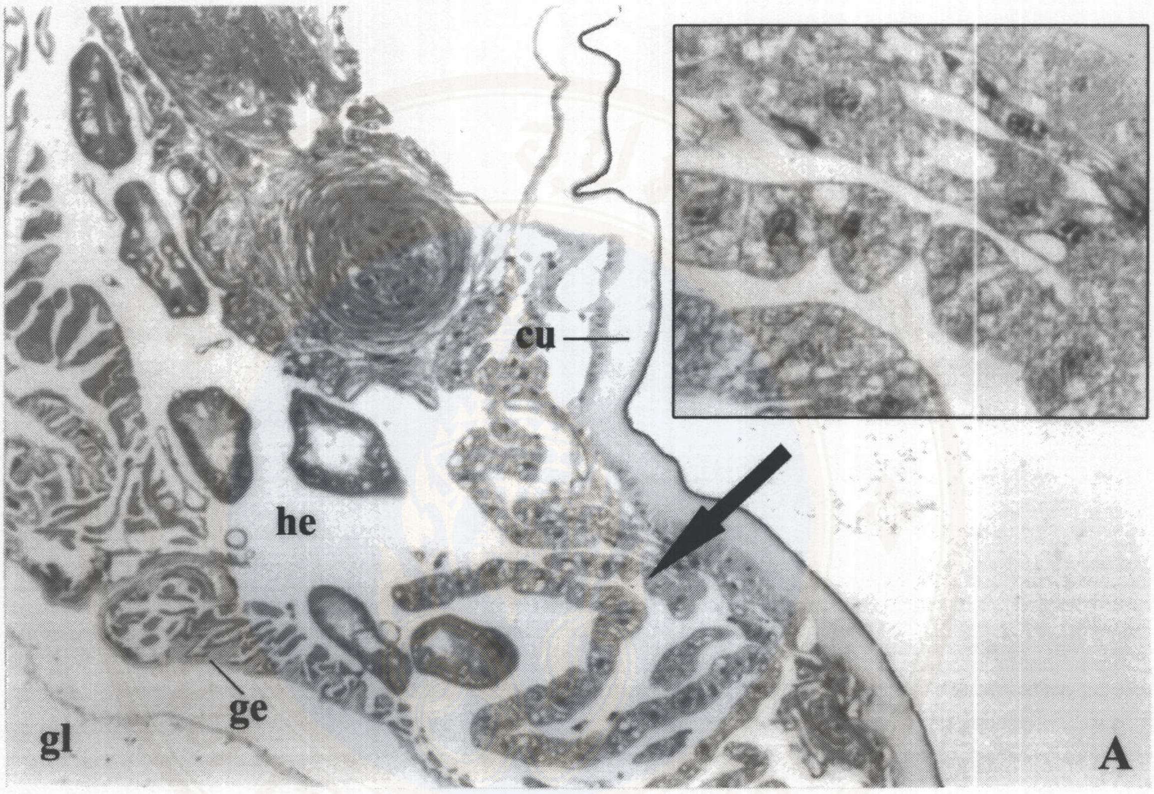

Figure 8. A longitudinal section of non-infected larva of *S. litura*. The specimen was stained with hematoxylin-eosin and examined under light microscope.

A: A section through the gut at the magnification of 100x (inset = a picture at higher magnification, 400x, of the area around the arrow). Both hyphae and hyphal bodies were not detected in the section of non-infected larva.

cu = Cuticle

he = Haemocoel

ge = Gut epithelial cell

gl = Gut lumen

epd = Epidermis

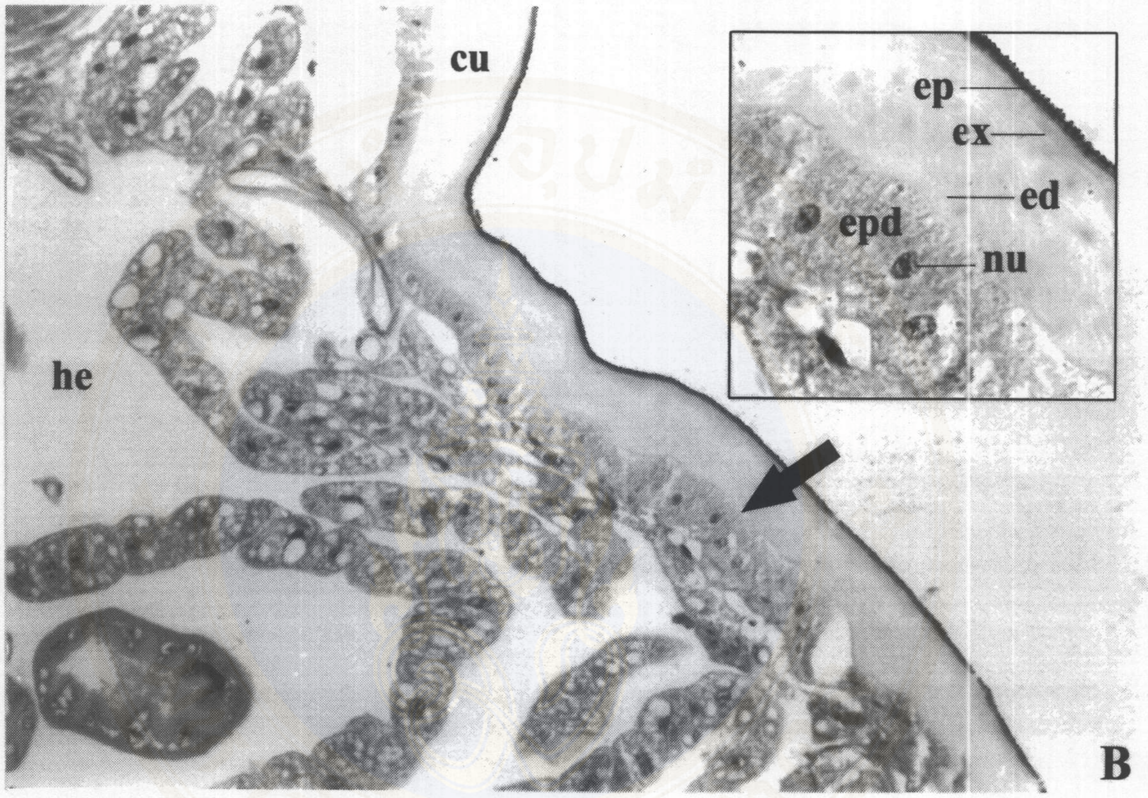

Figure 8. (Continue) B: A longitudinal section of non-infected larva of *S. litura* that was examined under the light microscope at the magnification of 200x (inset = a picture at higher magnification, 500x, of the area around the arrow).

ep = Epicuticle

ex = Exocuticle

ed = Endocuticle

epd = Epidermis

nu = Nucleus

he = Haemocoel

cu = Cuticle

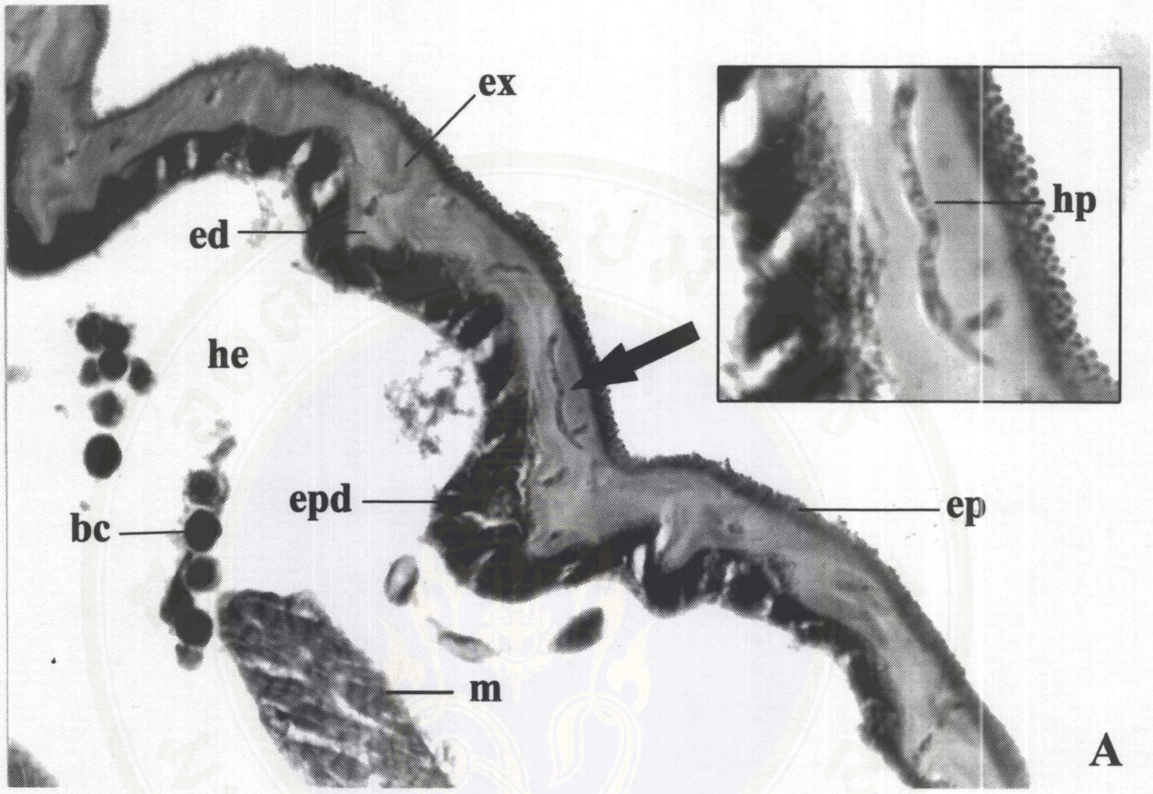

Figure 9. A longitudinal section of infected larva of *S. litura* that examined under the light microscope at the magnification of 400x (inset = a picture at higher magnification, 1000x, of the area around the arrow). The specimen was made at 2 days after inoculation and stained with H&E staining.

A: A picture shows laterally branched hyphae that grew along endocuticle laminae, between exocuticle and epidermis

ep = Epicuticle	bc = Blood cell
ex = Exocuticle	m = Muscle
ed = Endocuticle	hp = Hyphal
epd = Epidermis	he = Haemocoel

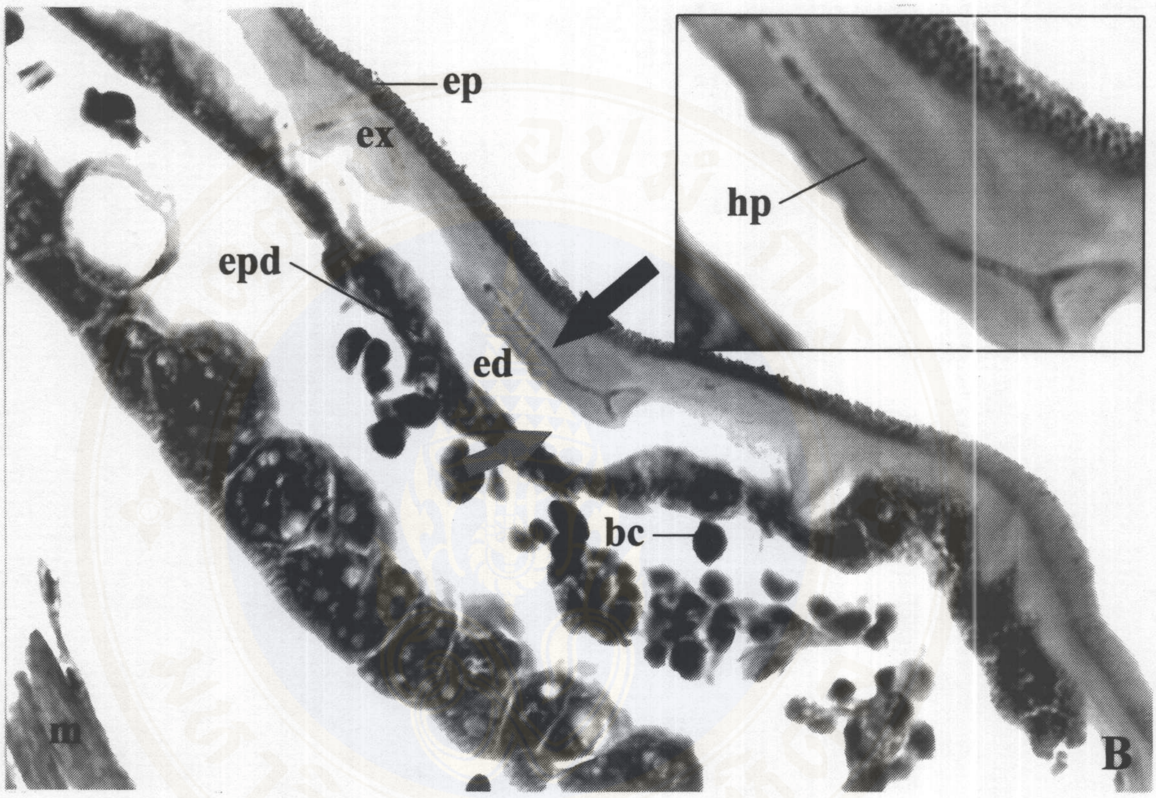

Figure 9. (Continue) A longitudinal section of infected larva of *S. litura* that examined under the light microscope at the magnification of 400x (inset = a picture at higher magnification, 1000x, of the area around the arrow).

B: A picture shows the lysis of endocuticle after the invasion of hypha. Red arrow indicates the lysis zone.

ep = Epicuticle	bc = Blood cell
ex = Exocuticle	m = Muscle
ed = Endocuticle	hp = Invasive hypha
epd = Epidermis	

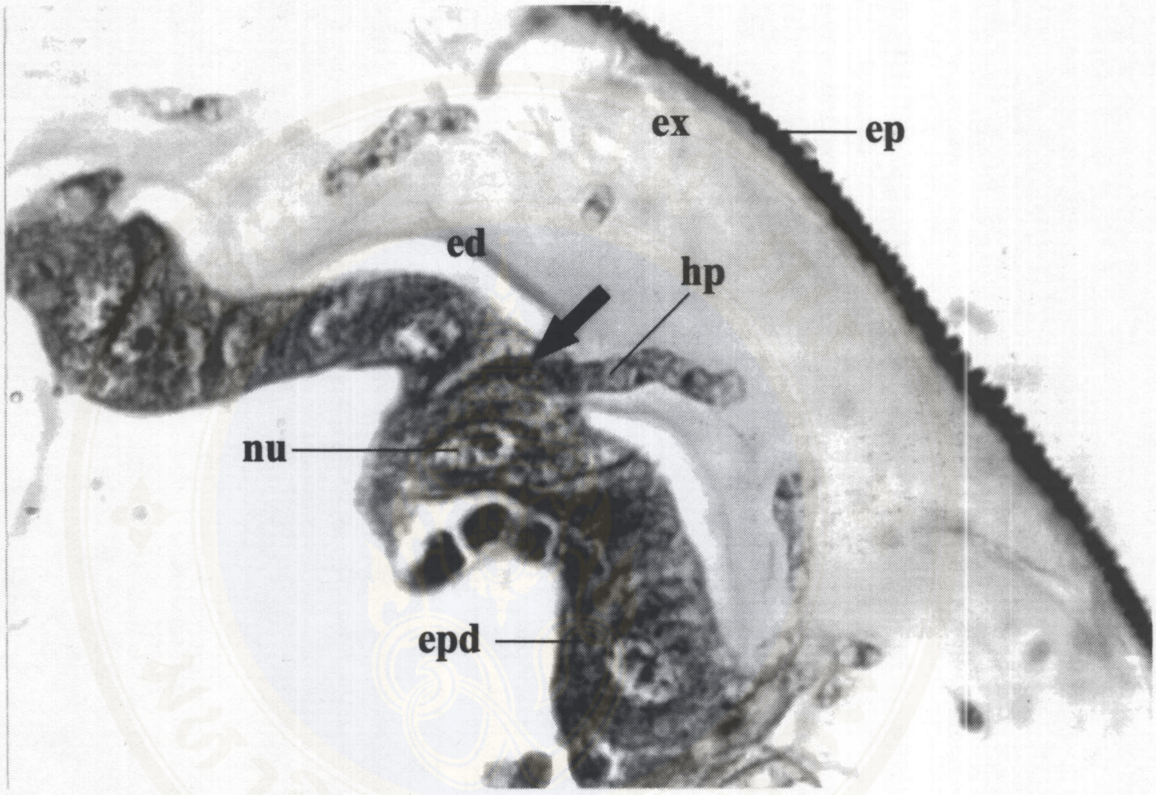

Figure 10. A longitudinal section of infected larva of *S. litura*. The specimen was made at 2.5 days after inoculation with the fungal isolate. A picture shows fungal hypha penetrating the epidermis and grew into the haemocoel. The specimen was examined under the light microscope at the magnification of 1000x. Arrow shows the point of penetration.

hp = Invasive hypha

epd = Epidermis

he = Haemocoel

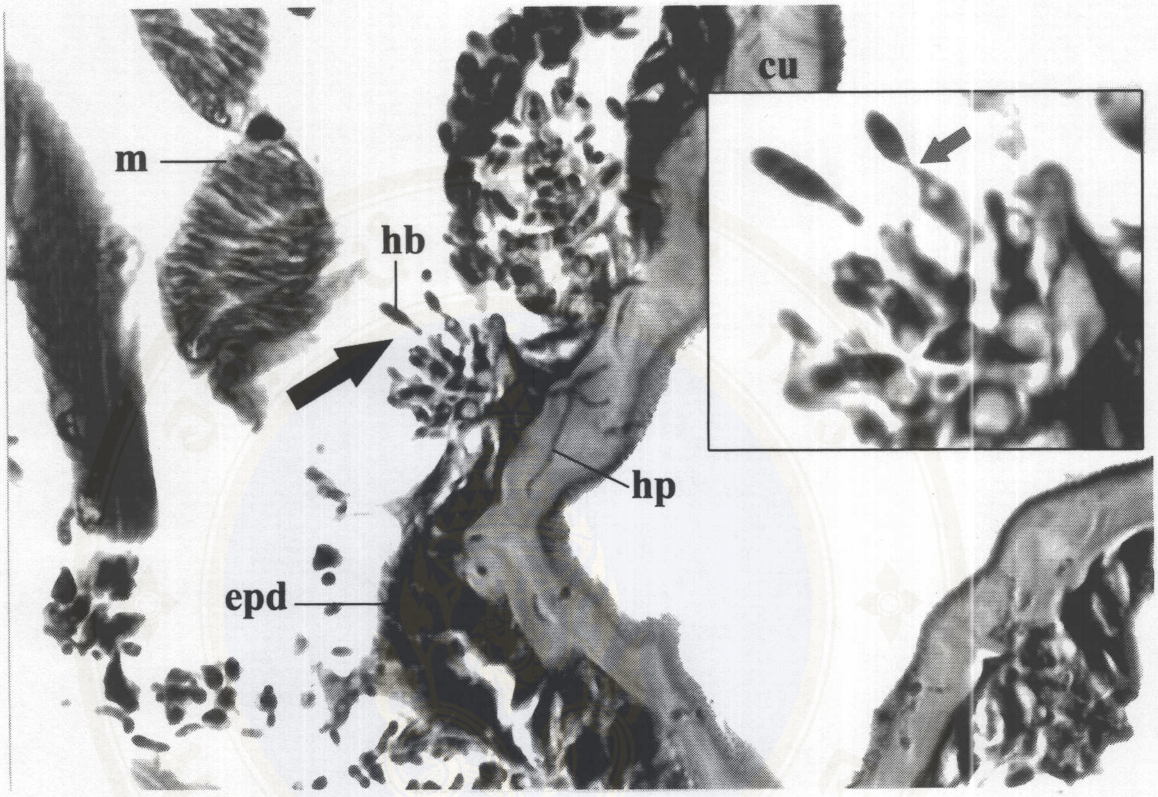

Figure 11. A longitudinal section of infected larva of *S. litura*. The specimen was made at 3 days after inoculation with the fungal isolate stained and examined under the light microscope at the magnification of 400x (inset = a picture at higher magnification, 1000x, of the area around the arrow).

A picture shows invasive hyphae switched into hyphal bodies and replicated by budding and septum formation in the haemocoel. Red arrow indicates the budding hyphal bodies.

hb = Hyphal bodies

hp = Hyphae

epd = Epidermis

he = Haemocoel

m = Muscle

cu = Cuticle

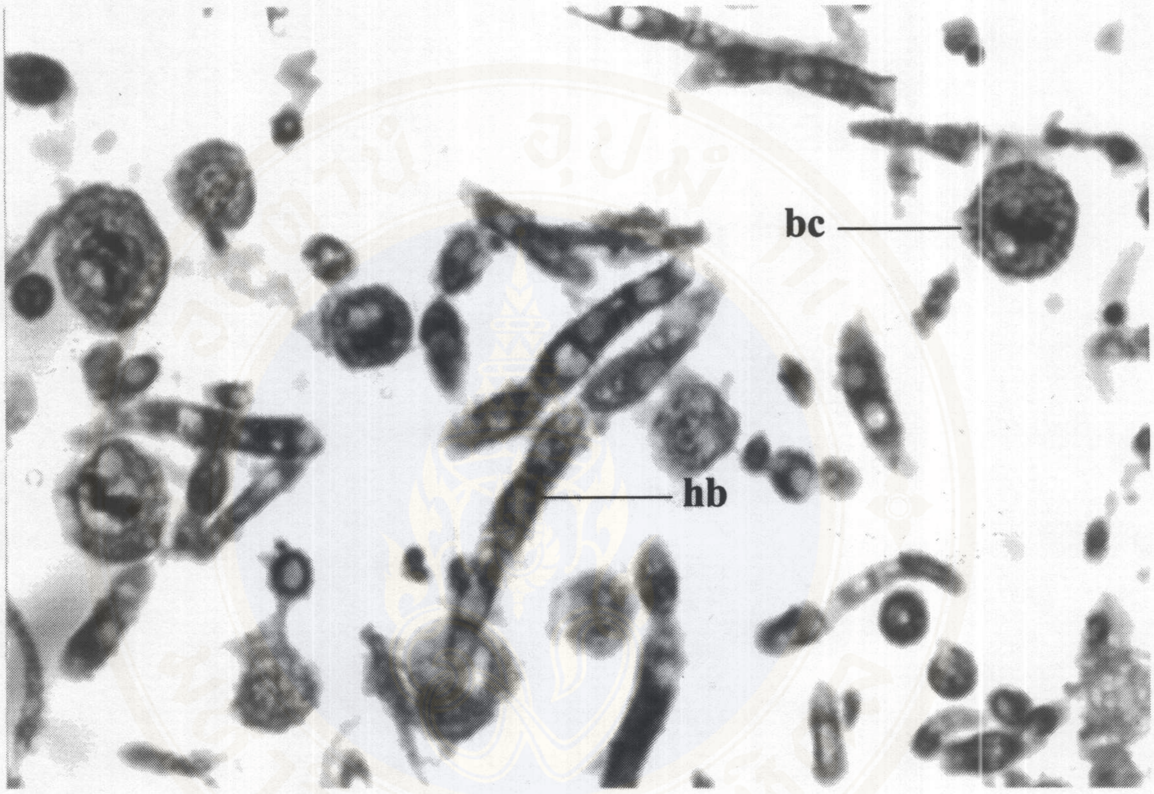

Figure 12 A longitudinal section of infected larva of *S. litura*. The specimen was made between 3.5 to 4 days after inoculation with the fungal isolate, stained and examined under the light microscope at the magnification of 1000x. A picture shows haemocoel was filled up with hyphal bodies and cohabited with blood cells.

bc = Blood cell

hb = Hyphal bodies

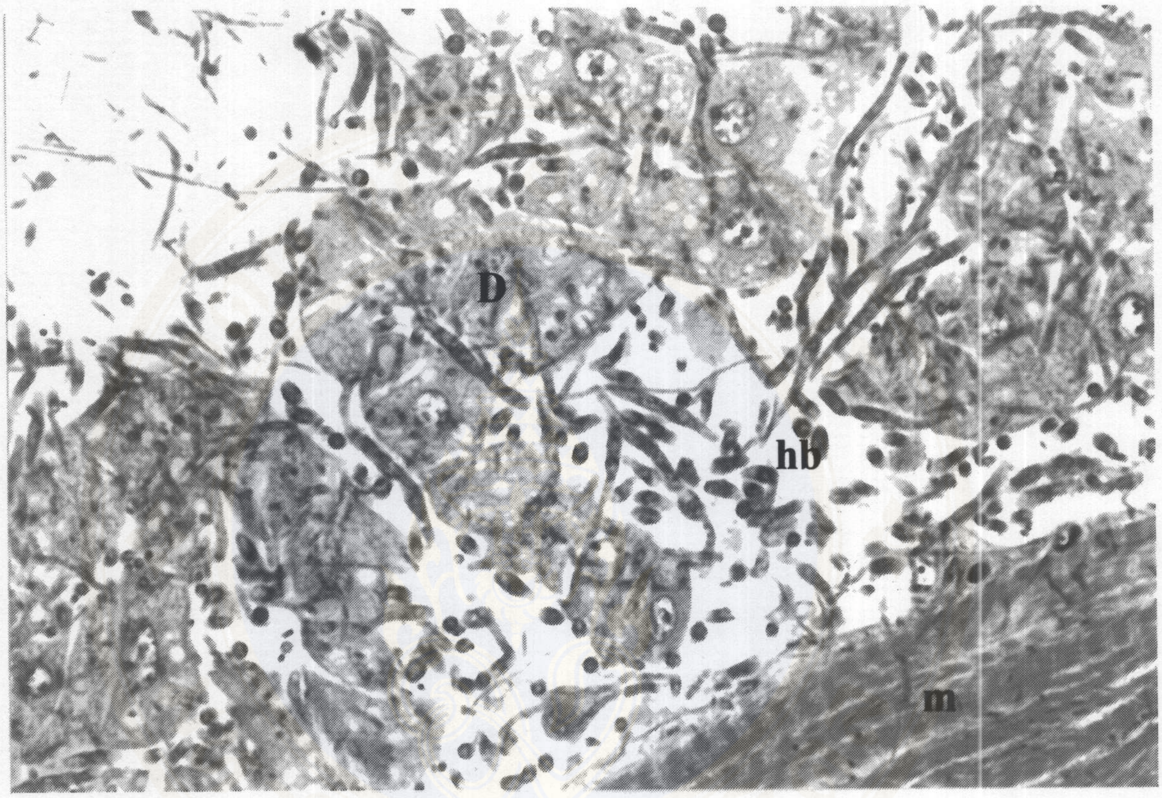

Figure 13. A longitudinal section of infected larva of *S. litura*. The specimen was made at 6.5 days after inoculation with the fungal isolate, stained and examined under the light microscope at the magnification of 200x. All tissues of infected larva were colonized with hyphal bodies.

D = Dermis

hb = Hyphal bodies

m = Muscle

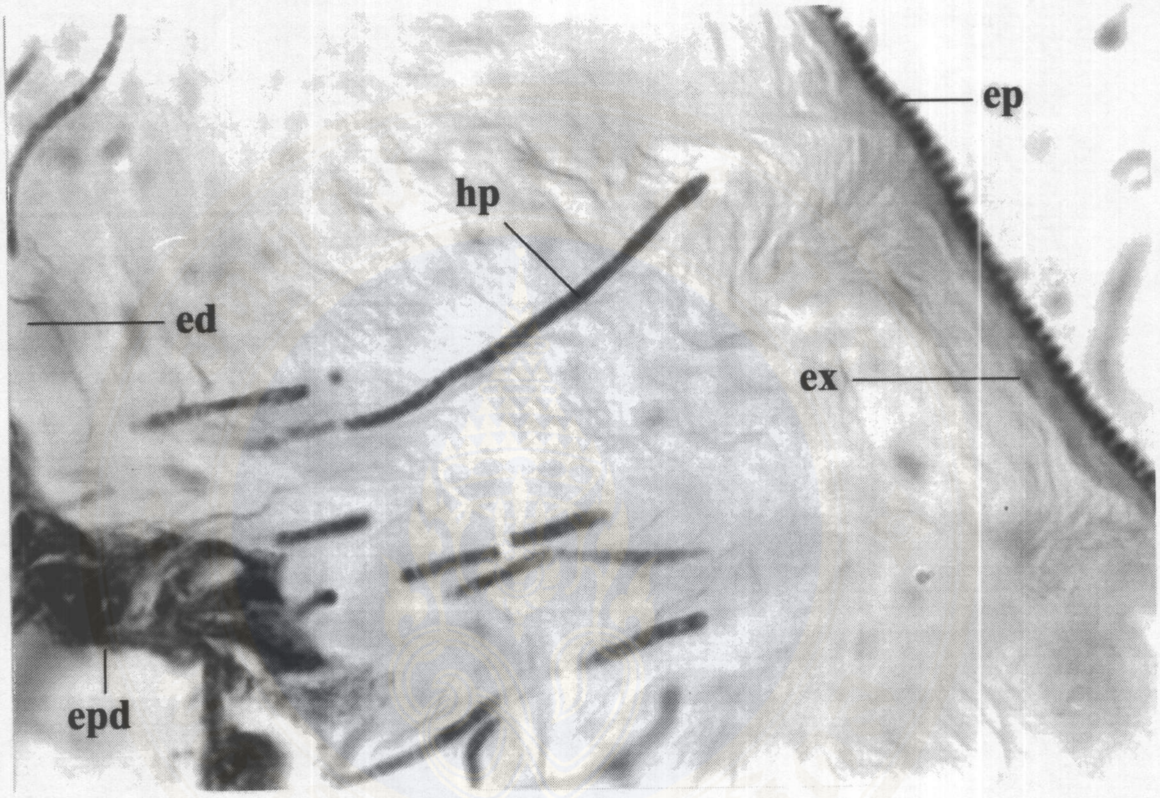

Figure 14 A longitudinal section of infected larva of *S. litura*. The specimen was made between 5.5 to 6 days after inoculation with the fungal isolate, stained and examined under the light microscope at the magnification of 1000x. A picture shows non-invasive hyphal bodies converted to invasive mycelia and penetrated through cuticle.

ep = Epicuticle

ex = Exocuticle

ed = Endocuticle

epd = Epidermis

hp = Hphae

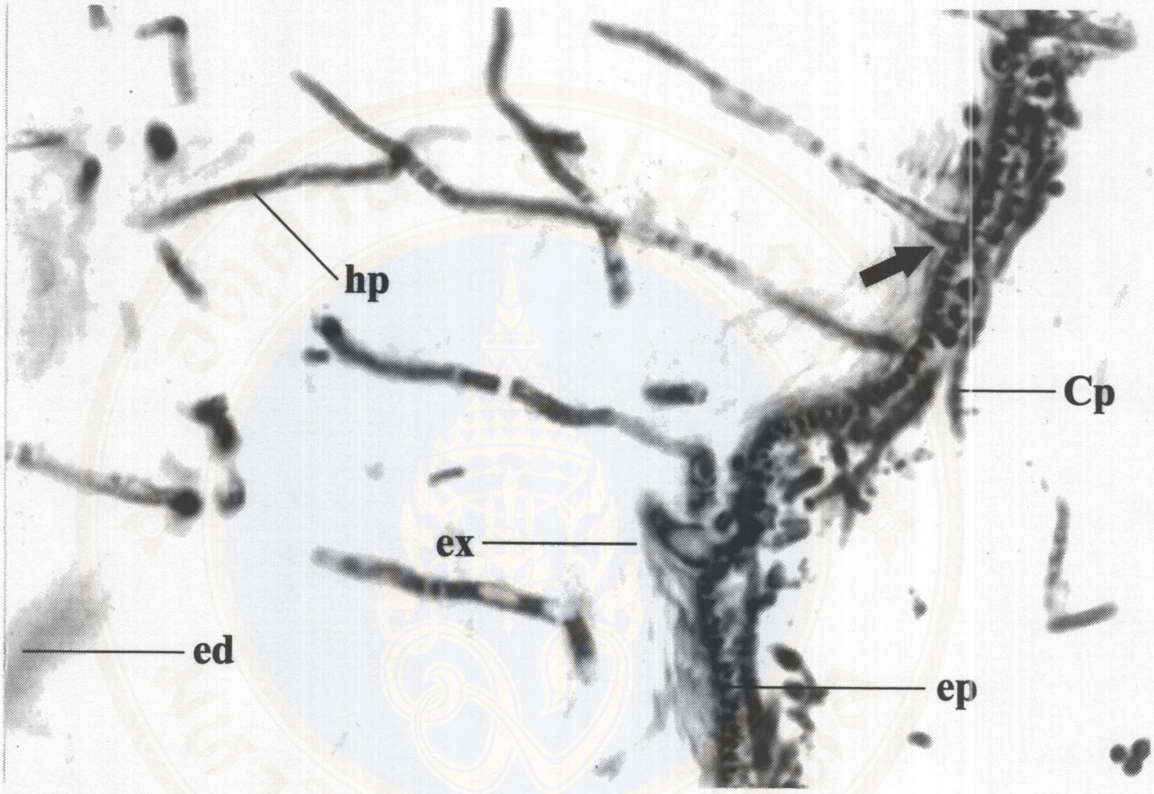

Figure 15. A longitudinal section of infected larva of *S. litura*. The specimen was made at 7 days after inoculation with the fungal isolate, stained and examined under the light microscope at the magnification of 1000x. Mycelia of the fungal isolate ramified throughout the cuticle Arrow shows the conidiophore in the process of protruding out from the cuticle.

Cp = conidiophore

ep = Epicuticle

ed = Endocuticle

ex = Exocuticle

hp = Hyphae

4. Toxicity Bioassay

Upon the mortality data of insect larvae showed that the dead larvae treated with different passages of conidia that had been transferred on FMAY were initially observed within 3- 4 days after inoculation, while those transferred on SMAY were initially observed within 5- 6 days after inoculation. The average LT_{50} values produced by conidia of the fungal isolate derived from FMAY and from SMAY medium were 6.16 and 7.62 days, respectively. The conidia derived from SMAY lost the ability to produce yeast-liked hyphal bodies after the 4th passage of serially transferring and the reduction in virulence was also observed at the same passage. The test larvae infected with conidia derived from FMAY medium could prolong their productivity of the green conidia on insect cadavers while those larvae infected with conidia derived from SMAY medium could not produce green conidia as shown in figure 18B. The deficiency in spore production was observed when conidia were obtained from the culture that had been serially subcultured from the 8th passage onward.

The LC_{50} values produced by three standard strains of *N. rileyi* were summarized in table 5 and the LC_{50} values produced by the fungal isolate cultured on FMAY and SMAY were summarized in table 6. These data indicated that the standard strain No. 5762 gave the lowest LC_{50} and LT_{50} values of 1.74×10^4 conidia/larva and 7.56 days, respectively. The highest LC_{50} and LT_{50} values produced by standard strain No. 5765 were 7.20×10^{10} conidia/larva and 10.83 days, respectively. The LC_{50} values produced by conidia derived from FMAY medium were approximately in the range of 910 conidia/larva to 42 conidia/larva. The LC_{50} values were fluctuated, which gave the maximum level at the 6th passages whereas the minimum level was found at the

13th passage. The LC_{50} values produced by conidia derived from SMAY medium increased and reached the highest level at the 12th passage. It was interesting to note that the LC_{50} values produced by the 12th passage, which was subcultured on FMAY medium, decreased to the level of 38 conidia/larva. Furthermore, the LC_{50} value produced by conidia derived from the 12th passages on SMAY medium were almost equivalent to the LC_{50} value of attenuated strain No 5762, which was 1.74×10^4 conidia/larvae. The comparison of the LC_{50} values produced by conidia derived from cultures grown on two media, FMAY and SMAY, using independent *t* test also gave significant difference ($t = -2.697$, $df = 11$, $P_{0.05} = 0.021$).

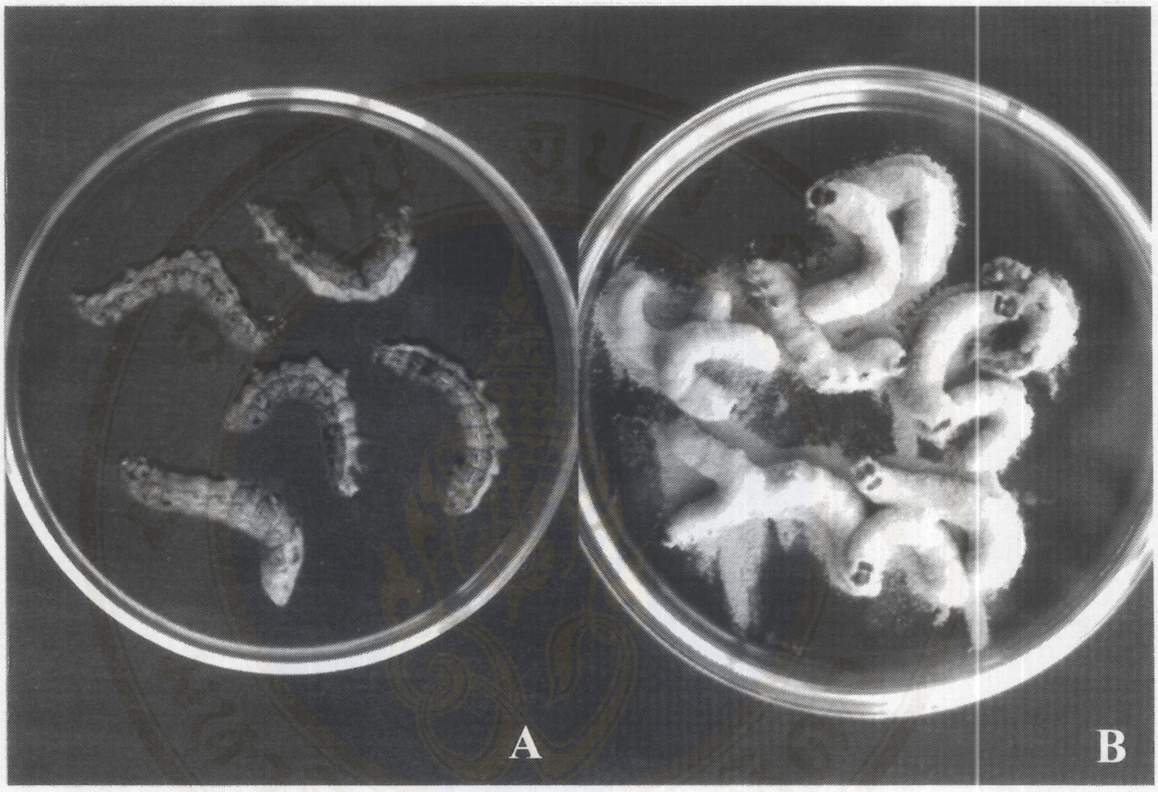

Figure 16. A: A picture shows paralyzed larvae of *S. litura* inoculated with the fungal isolate grown on FMAY medium. Symptom was observed on day 6 after inoculation. B: A picture shows infected larvae of *S. litura* was covered with green conidia after inoculating with the conidia of the fungal isolate grown on FMAY medium. The appearance of green conidia was observed on day 8 after inoculation.

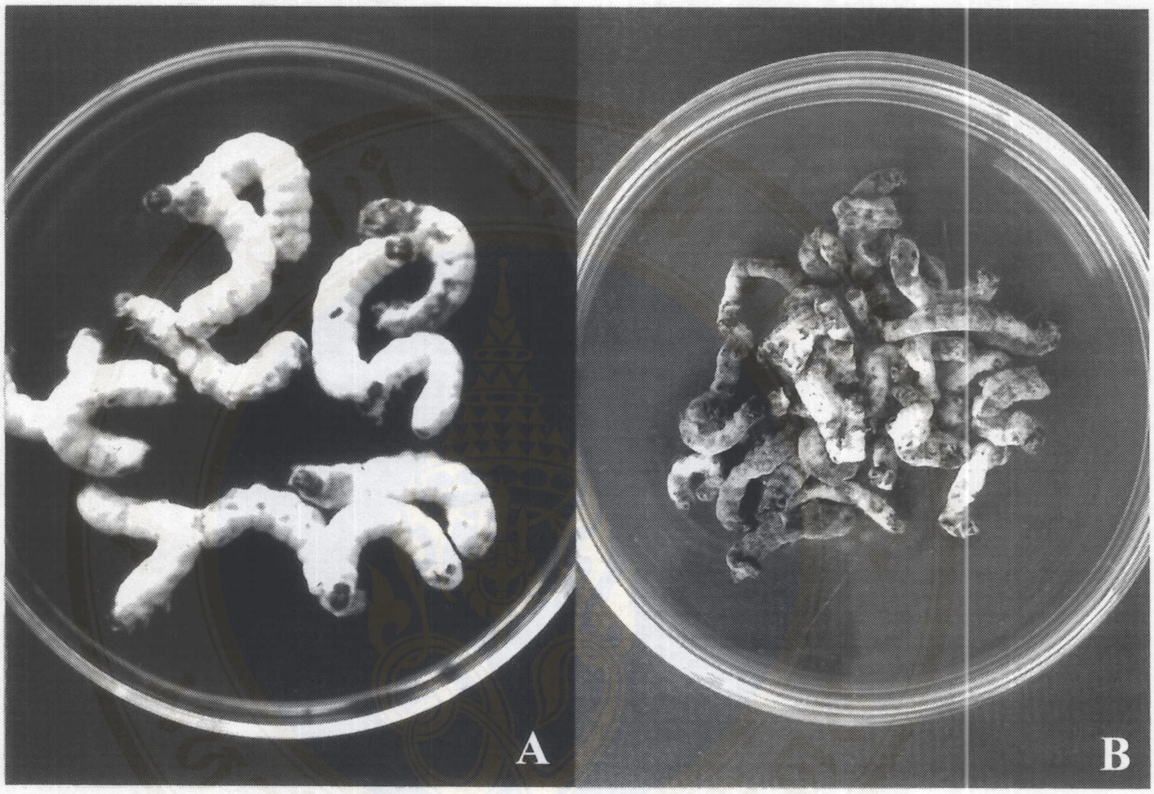

Figure 17. A: Infected larvae of *S. litura* were covered with the white mycelial mat after inoculating with conidia of the fungal isolate grown on FMAY on day 7 after inoculation. B: Infected larvae of *S. litura* were covered with the white mycelial mat after inoculating with conidia of the fungal isolate grown on SMAY on day 8 to 9. The fungal isolate could not produce the green conidia on insect cadavers. The bodies seemed more rigid and drier than the cadavers infected with conidia derived from FMAY medium.

Table 5. Estimation of the LC_{50} and LT_{50} values of standard strains of *N. rileyi* cultured on SMAY towards *S. litura*

Standard strains No.	**LT_{50} value (days)**	**LC_{50} value (spore/larva)**
5758	10.55	2.48x10^{6}
5762	7.56	1.74x10^{4}
5765	10.83	7.20x10^{10}

Table 6. Estimation of the LC_{50} and LT_{50} values produced by the conidia derived from FMAY(1st–15th passages) and conidia derived from FMAY (1st–12th passages) towards *S. litura*

Passage No.	**Conidia cultured on FMAY**		**Conidia cultured on SMAY**	
	LT_{50} value (days)	**LC_{50} value (spore/larva)**	**LT_{50} value (days)**	**LC_{50} value (spore/larva)**
1	5.70	202	7.07	7.26x10^{2}
2	5.73	178	6.33	1.97x10^{3}
3	4.70	138	8.47	3.66x10^{3}
4	5.01	259	7.27	1.52x10^{4}
5	5.65	81	7.79	1.30x10^{4}
6	6.29	910	7.13	1.11x10^{4}
7	8.03	344	7.79	2.10x10^{4}
8	7.34	680	7.46	2.42x10^{4}
9	6.91	70	8.05	3.99x10^{4}
10	6.26	24	7.42	4.75x10^{4}
11	6.60	169	8.27	7.88x10^{4}
12	6.56	46	8.33	1.44x10^{5}
13	6.55	42	N/A	N/A
14	5.48	83	N/A	N/A
15	5.53	148	N/A	N/A
Average values	6.156	225	7.615	3.34x10^{4}

Note: The toxicity was expressed in term of LT_{50} and LC_{50} calculated by probit regression by using software; SPSS for windows standard version (7.5).

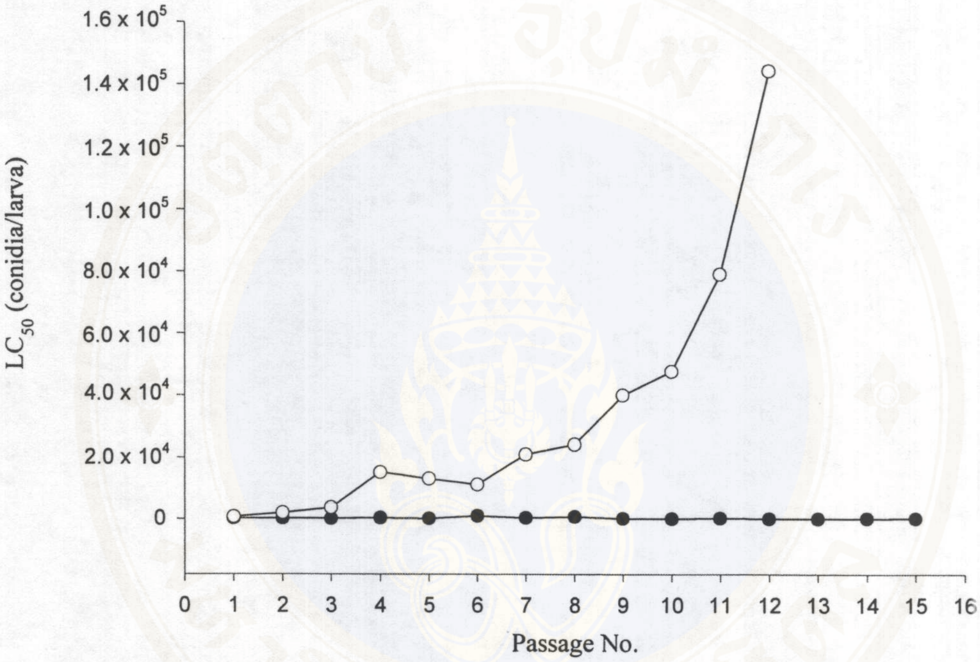

Figure 18. The LC_{50} values produced by conidia derived from the cultures grown on two media, FMAY and SMAY, were compared

(—○—) LC_{50} values produced by conidia derived from SMAY

(—●—) LC_{50} values produced by conidia derived from FMAY

5. Factors Attributing to the Virulence of the fungal isolate, *N. rileyi*

Growth rates of the fungal isolate

The comparisons between dry weights of the fungal isolate grown on FMAY medium and that grown on SMAY medium was carried out at every 3-day interval for 15 days. The dried cell weights were plotted against the incubation time and results are summarized in figure 19. It was found that fungal isolate grown on FMAY gave higher yields than that grown on SMAY medium. The difference between growth rates of the conidia grown on both media was observed at the 9th passage. The comparison of growth rate between different passages of conidia derived from FMAY and SMAY medium is shown in figure 20A-B, respectively. It was found that the capability of growth among these passages was proportional to the increasing time.

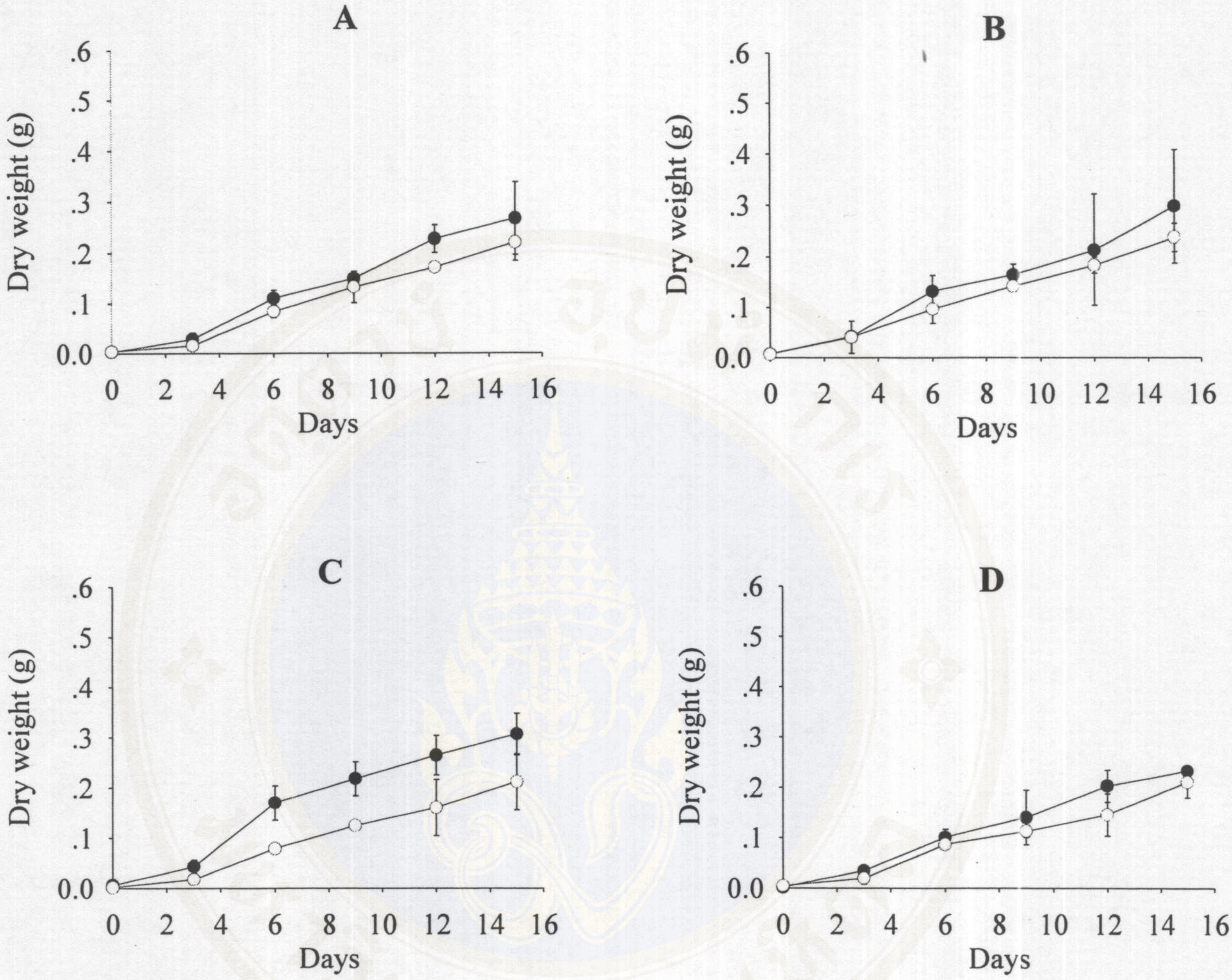

Figure 19. Comparison of growth rate of the 3rd, 6th, 9th and 12th passage of conidia derived from FMAY (—●—) and SMAY (—○—) medium. The conidia of each passage were harvested and inoculated into Sabouraud's maltose broth plus 1% yeast extract and incubated at 25°C on a shaker set at 150 rpm for 15 days. Each culture was taken every 3 days to determine the dried cell weight.

A = Growth rate of the 3rd passage on FMAY and SMAY medium

B = Growth rate of the 6th passage on FMAY and SMAY medium

C = Growth rate of the 9th passage on FMAY and SMAY medium

D = Growth rate of the 12th passage on FMAY and SMAY medium

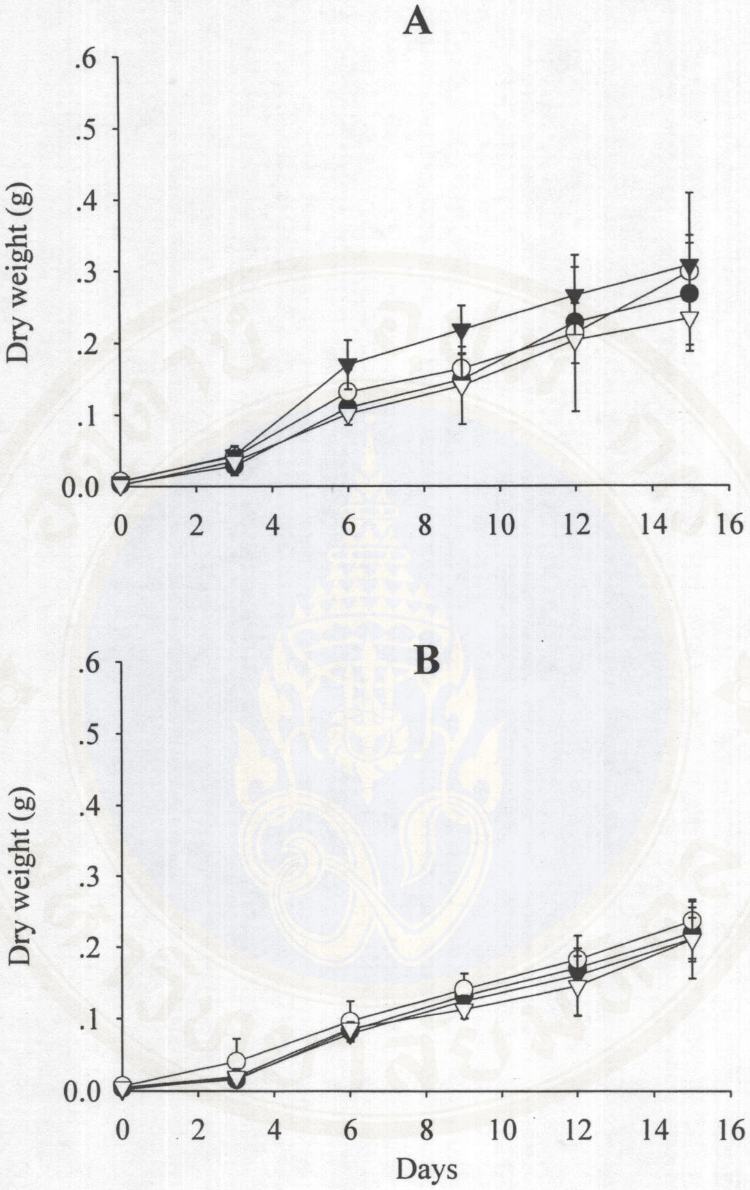

Figure 20. Comparison of growth rate at different passages of conidia derived from FMAY (A) and SMAY (B) medium.

—●— Growth rate of the 3rd passage
—○— Growth rate of the 6th passage
—▼— Growth rate of the 9th passage
—▽— Growth rate of the 12th passage

Germination of the conidia

Three hundred conidia were microscopically examined for the presence or absence of germ tubes. Results of the conidial germination of the fungal isolate derived from SMAY medium at various 12-day intervals are summarized in figure21. It was found that the germination of conidia derived from FMAY medium of the 3rd, 6th, 9th, 12th and 15th passages that observed on 24 and 36 h. germinated more rapidly and more abundant than those of conidia derived from SMAY medium of the 3rd, 6th, 9th and 12th passages. Results are summarized in table7. Since the estimation of conidial germination was performed only on filtered FMAY medium, this may affect the conidial germination of conidia derived from SMAY medium. Thus, the estimation of conidial germination was done with the same procedure on SMAY medium. It was interesting to note that the germination of conidia derived from SMAY medium grown on SMAY medium could not detected until 48 h. Furthermore, the conidial germination of the standard strain of *N. rileyi* was lower than the conidial germination of conidia derived from FMAY medium, but was still higher than the conidial germination of conidia derived from SMAY medium.

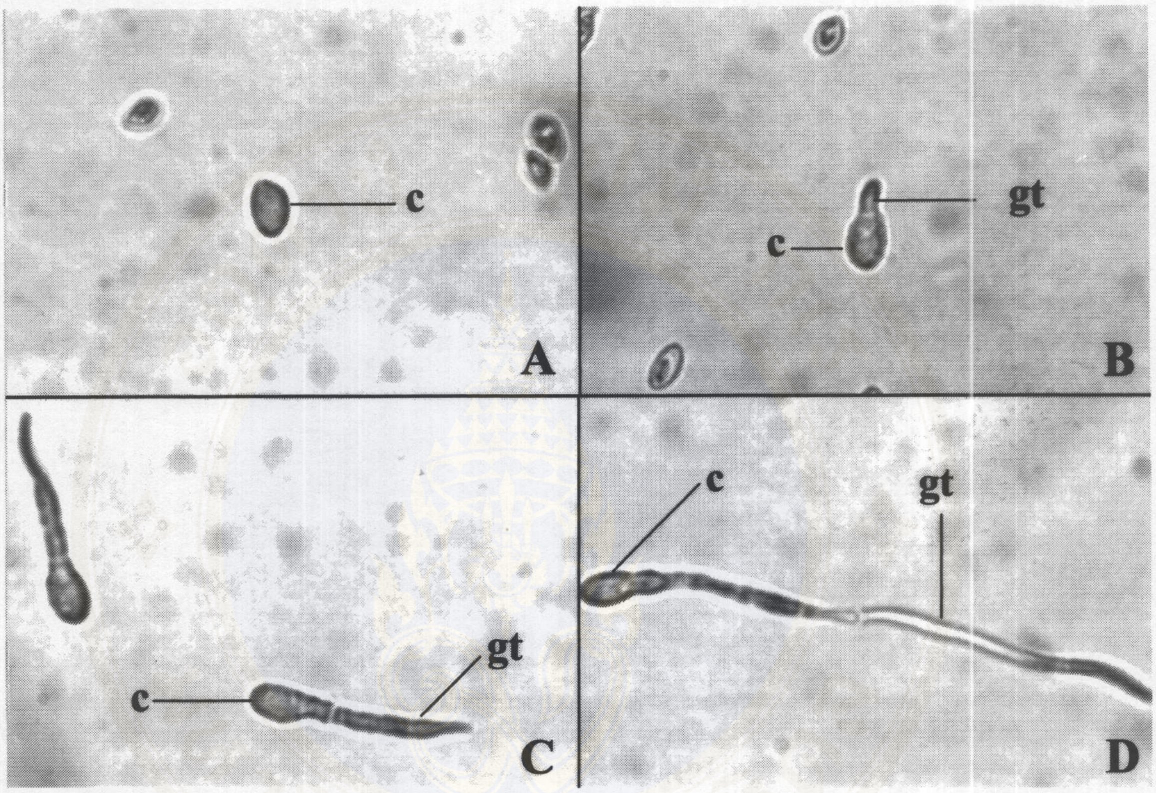

Figure 21. The germination of conidia of the fungal isolate. A picture showed the conidial morphology of the isolate germinating on SMAY medium at various time intervals. The pieces of agar were stained with Lacto-phenol cotton blue and were examined under light microscope at the magnification of 1000x.

A = The conidial germination at 0 h

B = The conidial germination at 24 h

C = The conidial germination at 36 h

D = The conidial germination at 48 h

C = conidium

gt = germ tube

Table 7. The comparison of the percentage germination of the fungal isolate of conidia derived from SMAY and FMAY medium with those of standard strain of *N. rileyi* at various time intervals.

Type of media for production	Passage No. of conidia	Percentage of germination of conidia test on					
		Filtered FMAY			SMAY		
		24h	36h	48h*	24h	36h	48h
FMAY	3	66.44	95.22				
	6	87.67	100				
	9	67.33	97.44				
	12	71.00	95.44				
	15	86.57	99.22				
	Average values	75.80	97.46				
SMAY	3	4.33	35.22		0	0	0.56
	6	4.56	42.44		0	0.11	0.67
	9	0.56	18.22		0	0.11	1.00
	12	0.33	14.67		0	0	0.22
	Average values	2.45	27.64		0	0.06	0.61
Standard strain No. 5758	-	17.33	72.67				
Standard strain No. 5765	-	31.22	83.56				
Standard strain No. 5768	-	7.78	44.67				

* **Note**. The conidial germination at 48 h on filtered FMAY was uncountable due to the overlapping of their germ tubes.

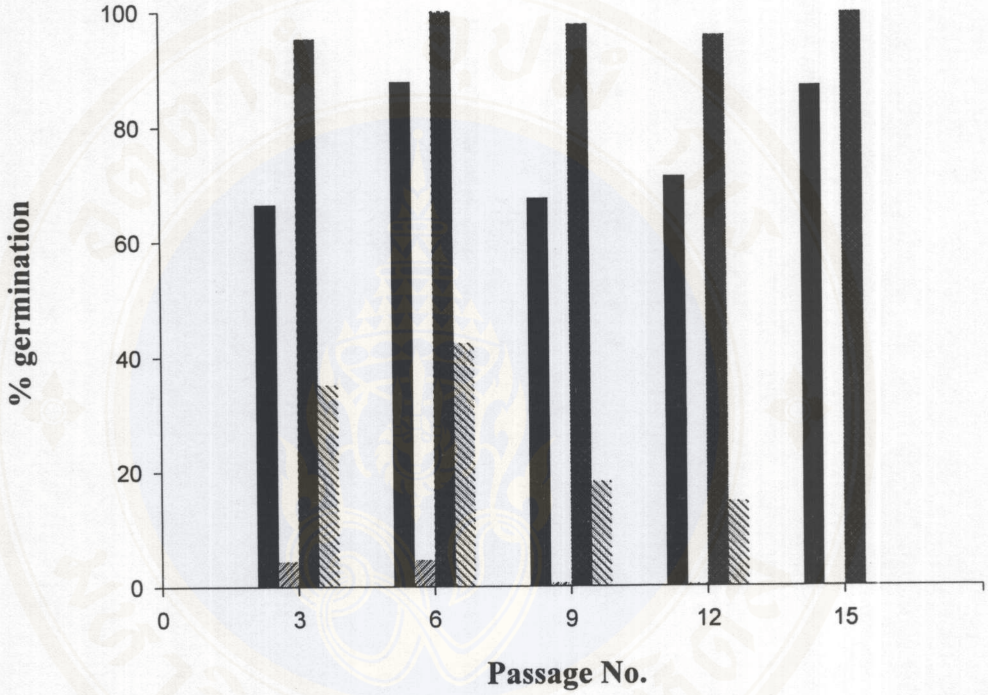

Figure 22. The comparison of percentage of conidial germination that derived from FMAY and from SMAY medium. The germination of conidia was done on filtered FMAY medium.

■ % germination of conidia derived from FMAY at 24 h.
▨ % germination of conidia derived from SMAY at 24 h.
■ % germination of conidia derived from FMAY at 36 h.
▧ % germination of conidia derived from SMAY at 36 h.

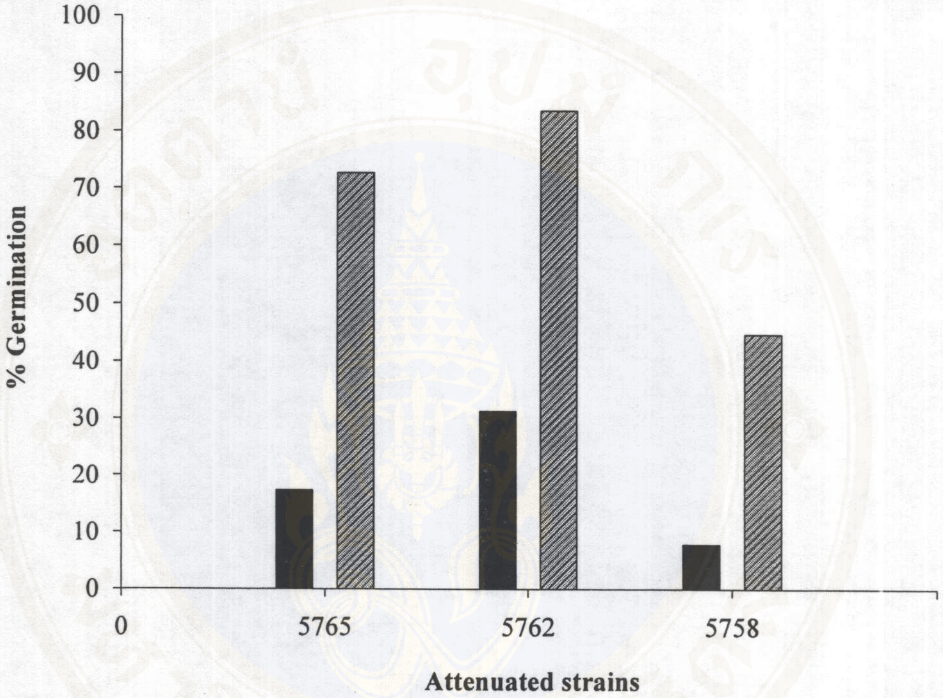

Figure 23. The comparison of percentage of conidial germination of standard strains 5765, 5762 and 5758, of *N. rileyi* germinating on filtered FMAY.

■ % germination of conidia at 24 h.
▨ % germination of conidia at 36 h.

Determination of spore production in artificial medium

The decrease in spore production during serial transfer of conidia derived from SMAY medium was observed. The deficiency of spore productivity was found after the 4th passage of serially transfer of conidia derived from SMAY medium. The spore production during the 3rd-15th passages of conidia derived from FMAY medium was initially observed within 6 days in the range of 10^7-10^8 whilst the spore production during the 3rd -12th passages of conidia derived from SMAY medium was initially observed within 9-15 days in the range of 10^5-10^7. The rate of sporulation of conidia derived from SMAY medium was slower those of conidia derived from FMAY medium. The yield of conidia from the 6th–12th passages of conidia derived from SMAY medium was lower than those of conidia derived from FMAY medium.

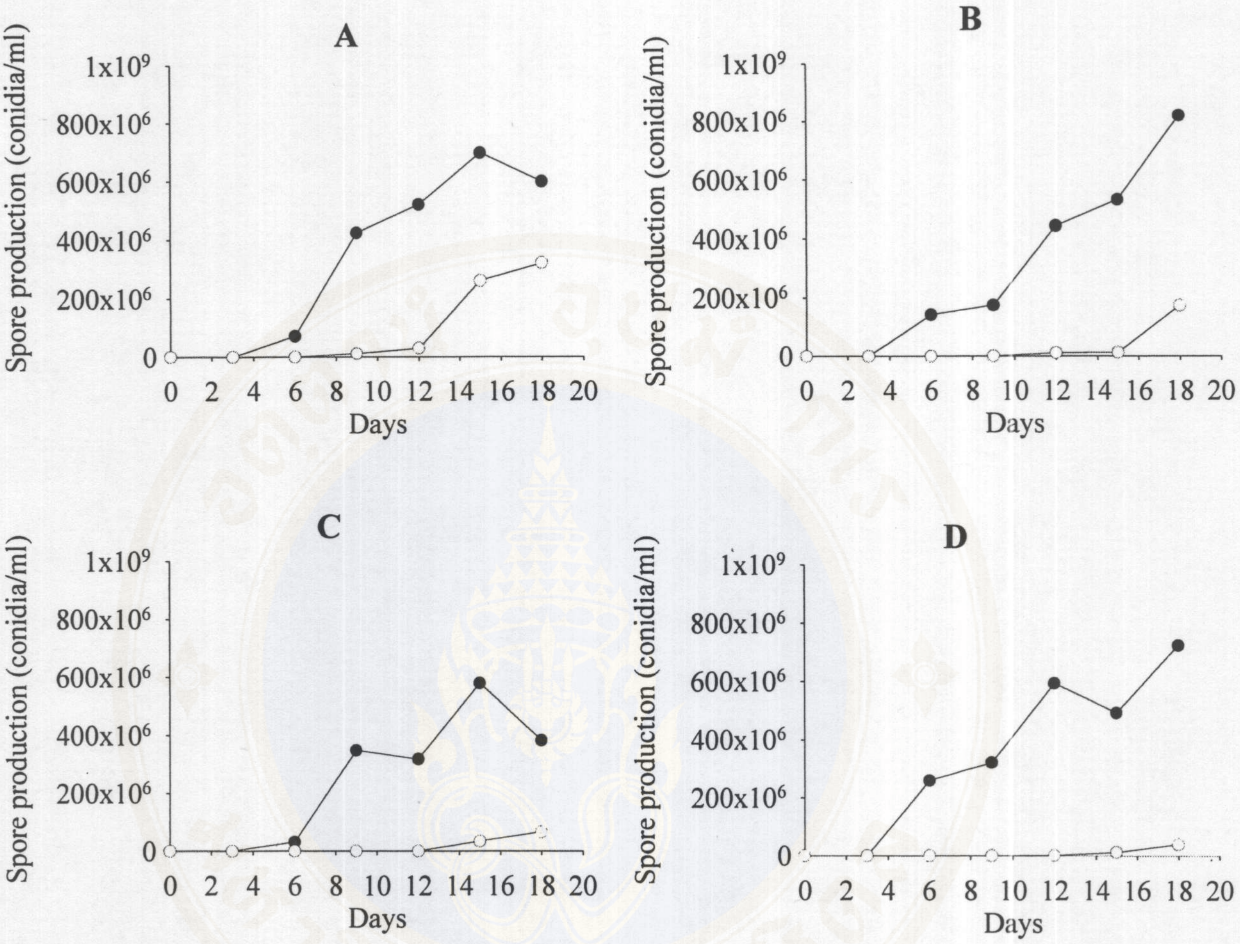

Figure 24. Comparison of sporulation of conidia derived from FMAY (—●—) and from SMAY(—○—) medium at the same passage for 18 days. The conidia were harvested and counted on every three days.

A = Spore production of the 3rd passage on FMAY and SMAY medium

B = Spore production of the 6th passage on FMAY and SMAY medium

C = Spore production of the 9th passage on FMAY and SMAY medium

D = Spore production of the 12th passage on FMAY and SMAY medium

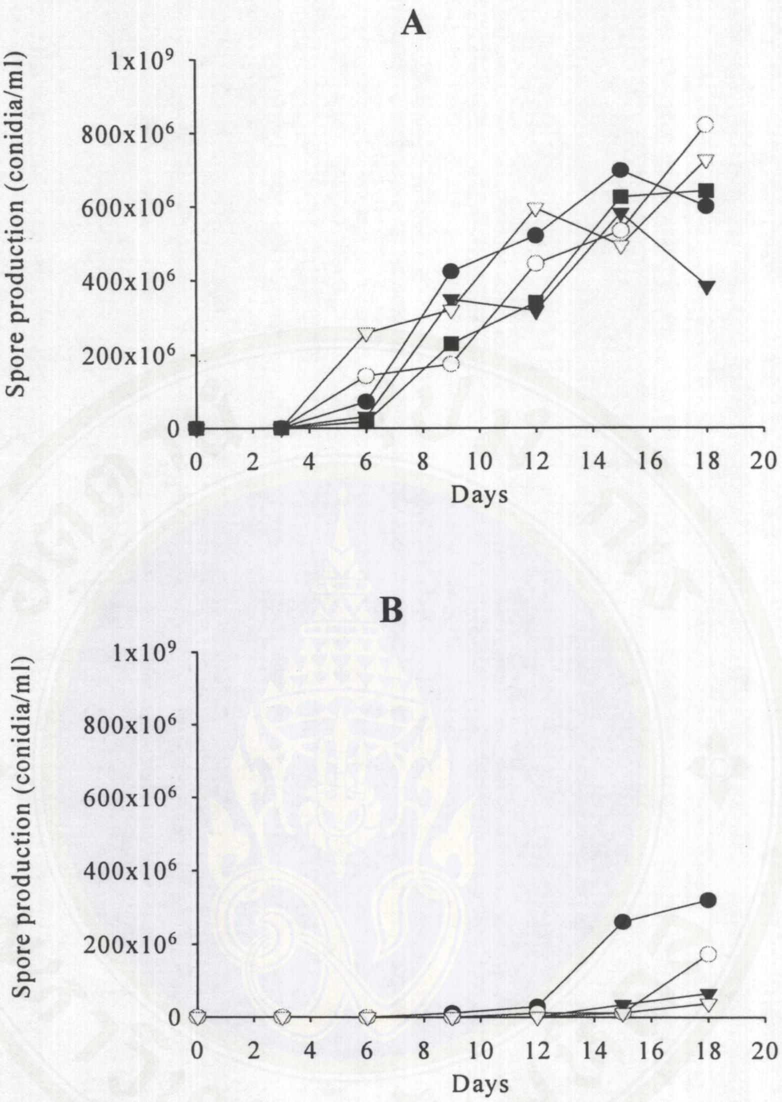

Figure 25. A: The comparison of sporulation among different passages of conidia derived from FMAY medium grown on FMAY medium. B: The comparison of sporulation among different passages of conidia derived from SMAY medium grown on SMAY medium.

—●— Spore production of the 3rd passage
—○— Spore production of the 6th passage
—▼— Spore production of the 9th passage
—▽— Spore production of the 12th passage
—■— Spore production of the 15th passage

6. Determination of Fish Soluble Components

The crude fat and fatty acid profiles were obtained by Soxhlet and gas liquid chromatography (GLC) methods, respectively. The fatty acid profile of Tuna soluble extract was shown in table 8. Total fat of tuna soluble extract was 36 mg/g. The highest fatty acid was Palmitic acid with approximate 29.5% of total fatty acid. Oleic acid and Docosahexaenoic acid were found to be approximately 17.4 and 13.1 % of total fatty acid, respectively. The percentages of these three fatty acids were significantly higher than other fatty acids. Other fatty acids that containing carbon atom lower than 10 atoms were not detected in tuna soluble extract.

Table 8. Fatty acid profile of Tuna soluble extract (Fish soluble medium)

Fatty acid	Structure of fatty acid	% of total fatty acid	weight
Caprotic acid	C6:0	0	0
Caprylic acid	C8:0	0	0
Capric acid	C10:0	0	0
Lauric acid	C12:0	0.3	0.01
Myristic acid	C14:0	5.4	0.14
Myristoleic acid	C14:1	0.2	0.01
Palmitic acid	C16:0	29.5	0.78
Palmitoleic acid	C16:1	5.7	0.15
Stearic acid	C18:0	9.5	0.25
Oleic acid	C18:1	17.4	0.46
Linoleic acid	C18:2	1.8	0.05
Gamma linoleic acid	C18:3, n6	1	0.02
Linolenic acid	C18:3, n3	0.8	0.02
Arachidic acid	C20:0	0.8	0.02
Eicosenoic acid	C20:1	2.1	0.05
Eicsadienoic acid	C20:2	0.3	0.01
Eicosatrienoic acid	C20:3	2.6	0.06
Arachidonic acid	C20:4	0.4	0.01
Eicosapentaenoic acid	C20:5	0.6	0.01
Behenic acid	C22:0	3.7	0.1
Erucic acid	C22:1	1.1	0.03
Docosahexaenoic acid	C22:6	13.1	0.35
Lognoceric acid	C24:1	1.2	0.03
Nervonic acid	C24:1	0.8	0.02

Note Total fat 3.6 g/100 g of tuna soluble extract

7. Determination of Crude Protein of the fungal isolate

The protein contents of conidia derived from various sources, i.e., the 2nd passage of conidia derived from FMAY, 12th passage of conidia derived from SMAY and standard strain no. 5765 of *N. rileyi*, were determined by Bradford assay using bovine serum albumin (BSA) as standard protein. The concentrations of these crude proteins are summarized in table 9. Protein patterns from all samples were not observed on days 3 and 4, but they were detected on day 5 post-inoculation. The highest amount of crude protein (1.63 mg/ml) was obtained from the conidia of standard strain no. 5765 that had been cultured for 5 days on SMBY medium. The amount of protein content was decreased to 0.72 mg/ml at 15 days after inoculation. The crude protein contents of conidia derived from the 2nd passage grown on FMAY medium were higher than that of the 12th passage grown on SMAY medium at 5 days after inoculation. The protein contents of conidia derived from the 2nd passage grown on FMAY medium decreased until they were about the same level with those of the 12th passage grown on SMAY medium after 15 days. All crude proteins were further analyzed by SDS-PAGE. Results of SDS-PAGE analysis are shown in figure 26. It was found that protein in lane 2 and lane 3, which were protein derived from the identical strains of fungal isolate, *N. rileyi,* showed the different patterns of proteins which were not seen in lane 5 and lane 6. The size of these proteins was determined by plotting graph between log polypeptide molecular weights and relative mobility on SDS-PAGE. The molecular weight of unknown A and B was found to be 43.0 and 25.04 kDa, respectively as shown in figure 27.

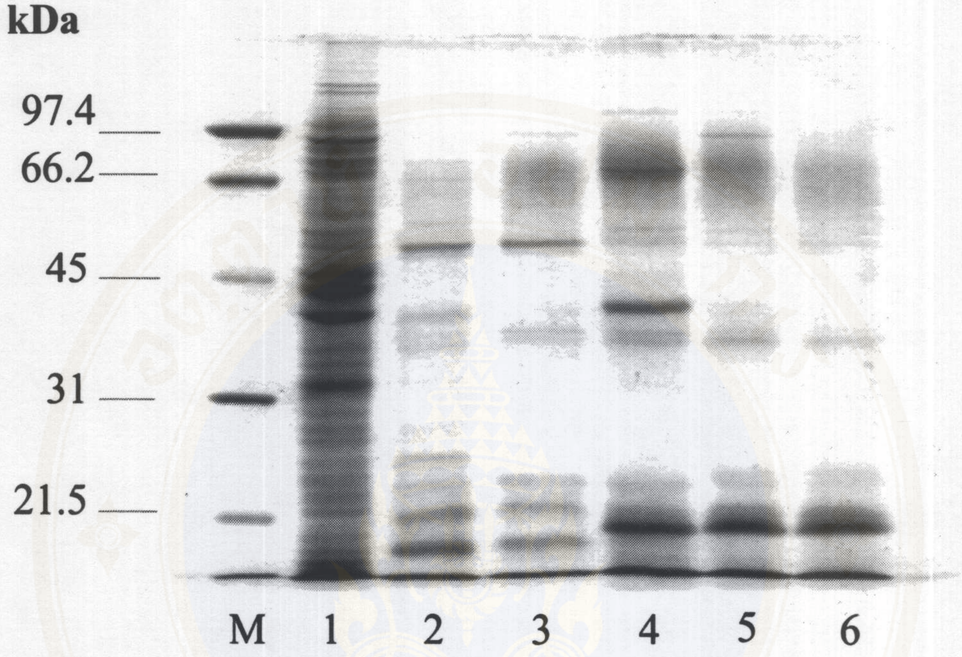

Figure 26. SDS-PAGE patterns of crude proteins from the supernatant of conidia derived from various sources. The conidia were cultured on SMBY and precipitating with 80% ammonium sulfate.

Lane 1 = Crude proteins from standard strain No. 5765 of *N. rileyi* after culturing for 5 days.

Lane 2 = Crude proteins from 2nd passage of conidia derived from FMAY after culturing for 5 days.

Lane 3 = Crude proteins from 12th passage conidia derived from SMAY after culturing for 5 days.

Lane 4 = Crude proteins from standard strain No. 5765 of *N. rileyi* after culturing for 15 days.

Lane 5 = Crude proteins from 2nd passage of conidia derived from FMAY after culturing for 15 days.

Lane 6 = Crude proteins from 12th passage conidia derived from SMAY after culturing for 15 days.

Lane M = Protein molecular weight standards, soybean trysin inhibitor (21.5 kDa), Carbonic anhydrase (31 kDa), Ovalbumin (45 kDa), bovine serum albumin (66 kDa) and Phosphorylase b (97.4 kDa).

Table 9. The concentrations of crude protein produced by *N. rileyi* derived from various sources of conidia. These conidia were cultured on Sabouraud's maltose broth (SMBY) for 5 and 15 days.

Conidial sources	Crude protein concentration (mg/ml)*	
	5-day incubation	15-day incubation
1) Standard strains No. 5765	1.63	0.72
2) 2nd passage of conidia derived from FMAY	0.79	0.58
3) 12th passage of conidia derived from SMAY	0.42	0.57

Note. Crude protein were obtained from the supernatant of various conidial sources cultured on SMBY and precipitating with 80% ammonium sulfate

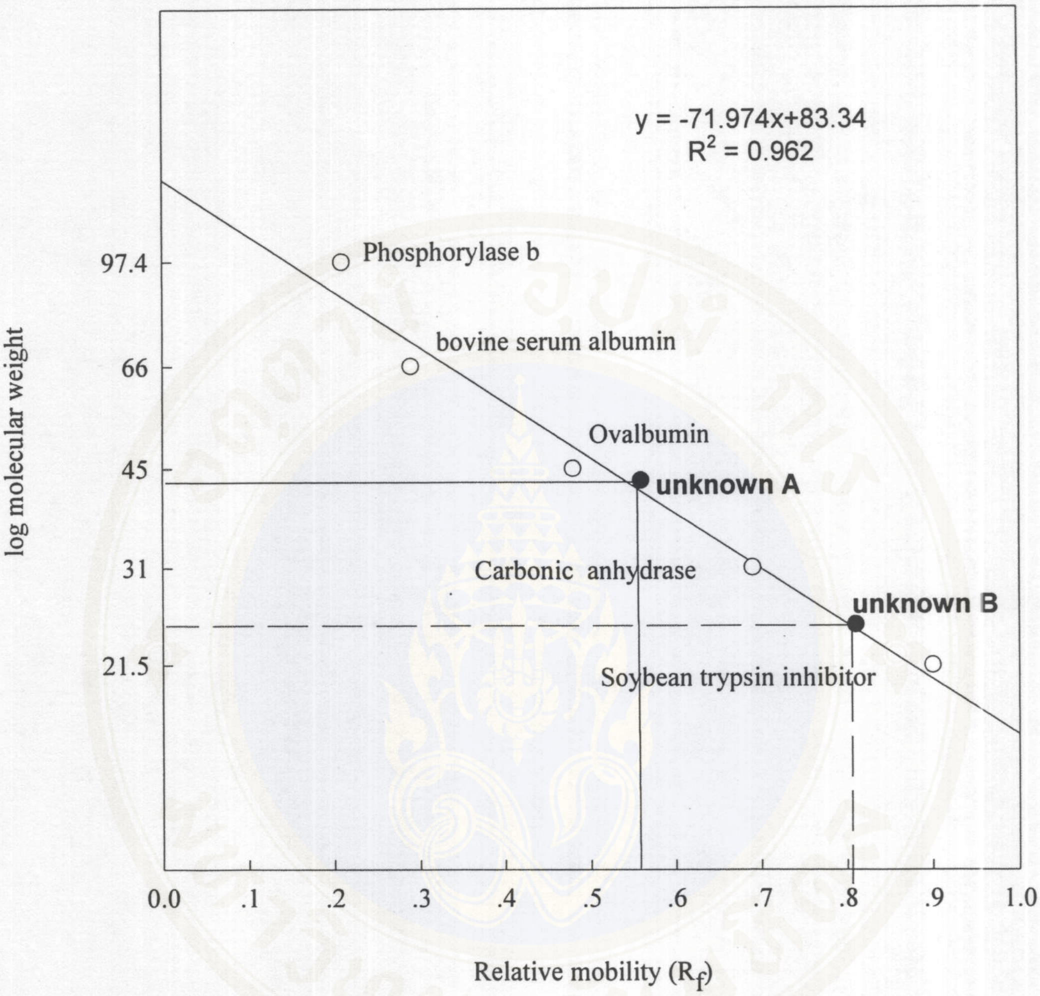

Figure 27. Determination of molecular weight of unknown A and B of crude proteins from the 2nd passage of conidia derived from FMAY after culturing for 5 days. The relationship between relative mobility (R_f) of standard proteins and log of molecular weight were determined by plotting of log polypeptide molecular weights versus relative mobility on SDS-PAGE. The molecular weights of unknown A and B were approximately 43 and 25.04kDa, respectively.

8. Determination of Cuticle-Degrading Enzymes Activity

The production of three extracellular cuticle-degrading enzymes was monitored during the growth of the fungal isolate in Sabouraud's maltose broth. The preliminary detection of chitinases, proteases and lipases activities in crude proteins was determined by the diffusion method and the results were shown in figure 28, 29, and 30, respectively.

Upon the chitinases detection, all crude proteins were further examined by the colorimetric method using colloidal chitin and glycol chitin as substrate Results are summarized in table 10. The lowest activities of chitinases were detected in crude proteins from standard strain No. 5765. However, the activities of chitinases from the 2nd and 12th passages of the fungal isolate were not significantly different.

Protease activities detected by diffusion method were negative in all samples of conidia derived from all sources, except that of the 2nd passage of conidia derived from FMAY medium. However, the proteases activity could be precisely detected by colorimetric method and its results showed that the highest activity was detected from the same sample.

Attempts were made to detect lipases activity and found that the lipase activities of all crude proteins were not different. All of them were approximately 400 -600 mU/mg. The lipase activity was considerably higher than other cuticle-degrading enzymes. The highest activity was detected from the 2nd passage of conidia derived from FMAY medium at 15th day after inoculation with the activity of 688 mU/mg.

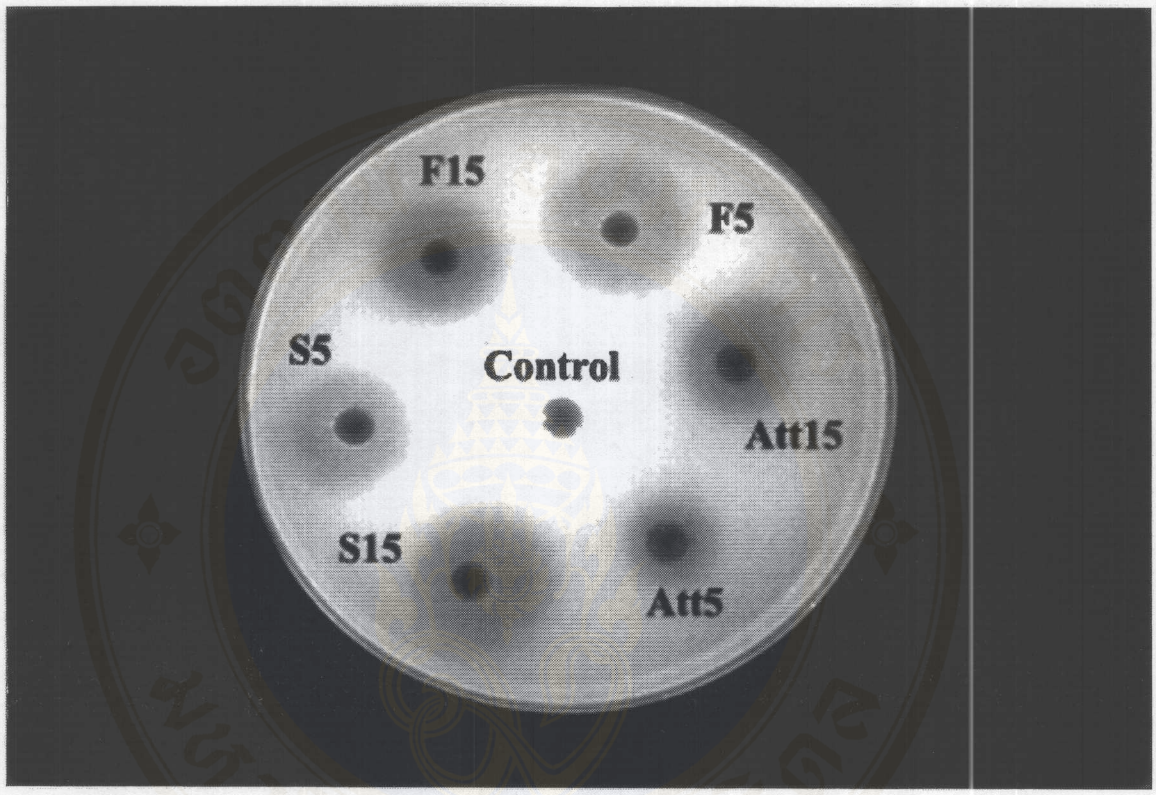

Figure 28. The activity of chitinases demonstrated by the diffusion method using the glycol chitin as substrate, the supernatant of crude protein was obtained from the 5th day and 15th day of the cultivation.

Att5 = Crude protein from standard strain no. 5762 of *N. rileyi* after culturing in SMBY for 5 days.
F5 = Crude protein of the 2nd passage of conidia derived from FMAY after culturing in SMBY for 5 days.
S5 = Crude protein from 12th passage of conidia derived from SMAY after culturing in SMBY for 5 days.
Att15 = Crude proteins from standard strain no. 5762 of *N. rileyi* after culturing in SMBY for 15 day.
F15 = Crude proteins from 2nd passage of conidia derived from FMAY after culturing in SMBY for 15 days.
S15 = Crude protein from 12th passage of conidia derived from SMAY after culturing in SMBY for 15 days.
Control = 0.01M Tris-HCl buffer pH 7.

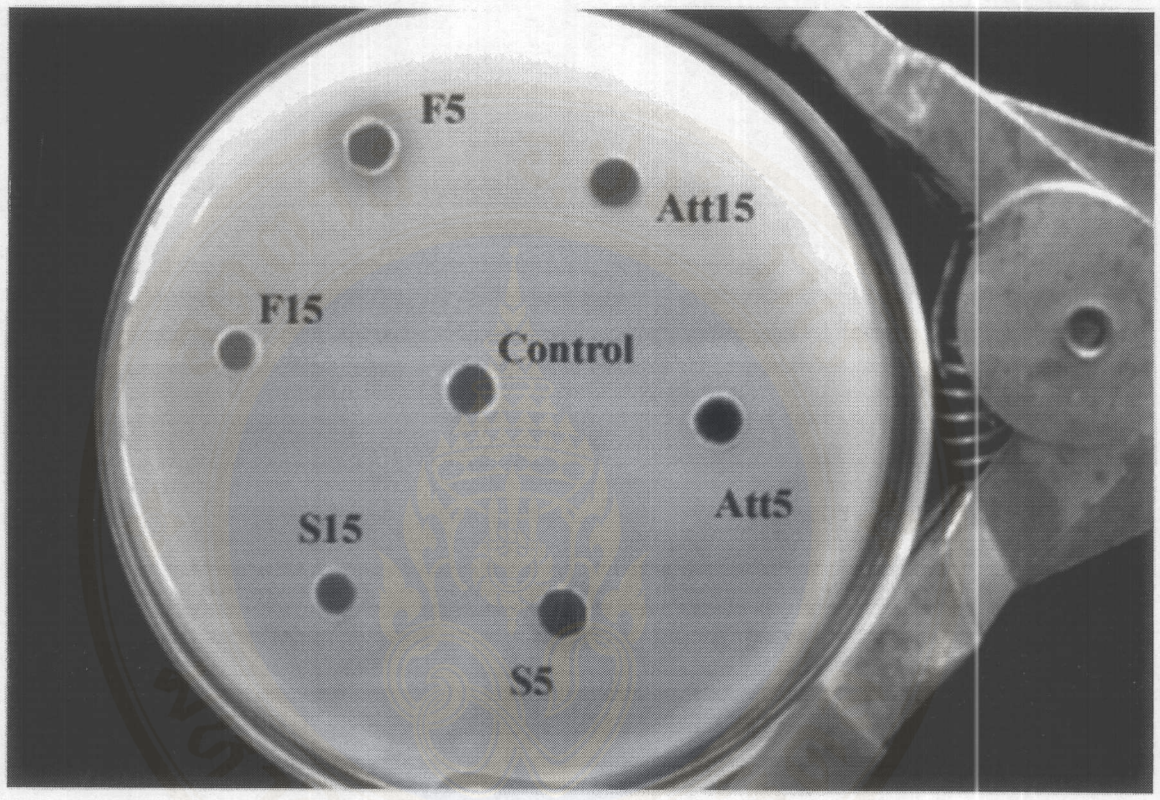

Figure 29. The activity of proteases demonstrated by the diffusion method using 1% skim milk as substrate, the supernatant of crude protein was obtained from the 5th and 15th day of the cultivation.

Att5 = Crude protein from standard strain no. 5762 of *N. rileyi* after culturing in SMBY for 5 days.

F5 = Crude protein of the 2nd passage of conidia derived from FMAY after culturing in SMBY for 5 days.

S5 = Crude protein from 12th passage of conidia derived from SMAY after culturing in SMBY for 5 days.

Att15 = Crude proteins from standard strain no. 5762 of *N. rileyi* after culturing in SMBY for 15 day.

F15 = Crude proteins from 2nd passage of conidia derived from FMAY after culturing in SMBY for 15 days.

S15 = Crude protein from 12th passage of conidia derived from SMAY after culturing in SMBY for 15 days.

Control = 0.01M Tris-HCl buffer pH 7.

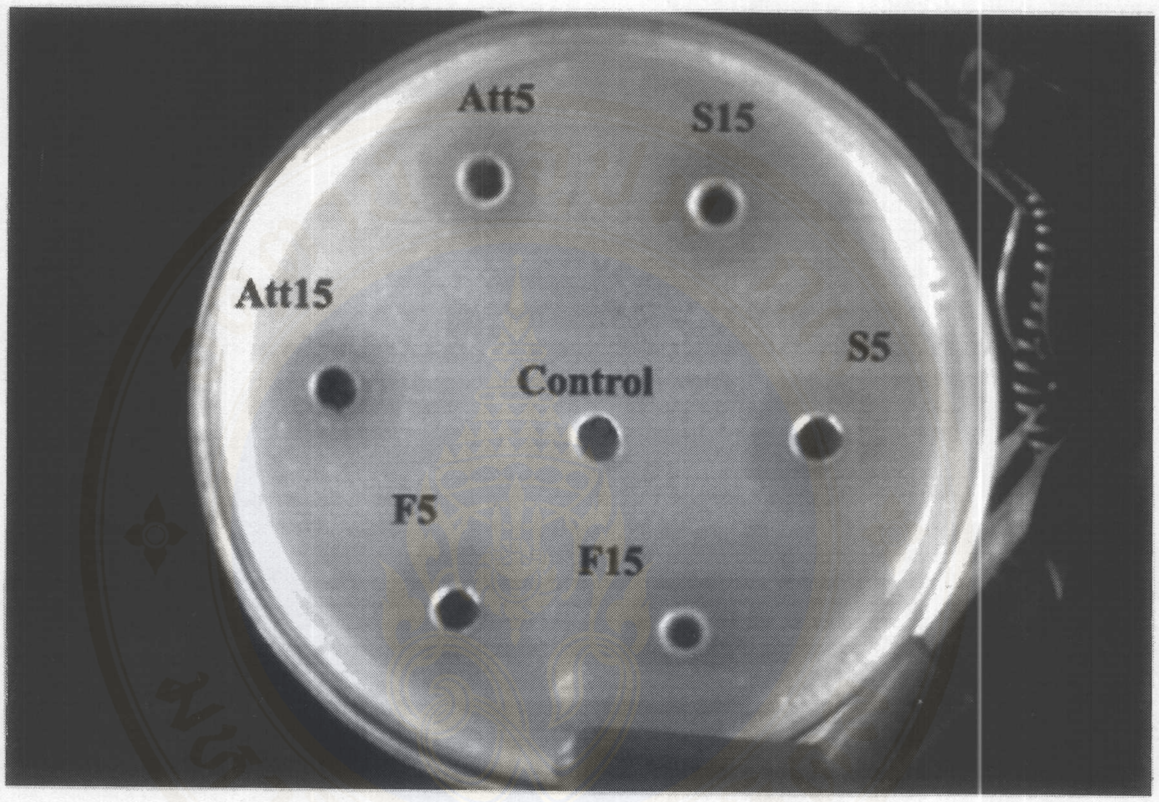

Figure 30. The activity of lipases demonstrated by the diffusion method using 1% tributyrin as substrate, the supernatant of crude protein was obtained from the 5th day and 15th day of the cultivation.

Att5 = Crude protein from standard strain no. 5762 of *N. rileyi* after culturing in SMBY for 5 days.

F5 = Crude protein of the 2nd passage of conidia derived from FMAY after culturing in SMBY for 5 days.

S5 = Crude protein from 12th passage of conidia derived from SMAY after culturing in SMBY for 5 days.

Att15 = Crude proteins from standard strain no. 5762 of *N. rileyi* after culturing in SMBY for 15 day.

F15 = Crude proteins from 2nd passage of conidia derived from FMAY after culturing in SMBY for 15 days.

S15 = Crude protein from 12th passage of conidia derived from SMAY after culturing in SMBY for 15 days.

Control = 0.01M Tris-HCl buffer pH 7.

Table 10. Estimation of the production of Cuticle-degrading enzymes during the conidial growth of *N. rileyi* in Saboraud's maltose broth at 5 days and 15 days from three conidial sources.

Conidial sources	**Specific activity of enzymes (mU/mg)**							
	Chitinases				**Proteases**		**Lipases**	
	Colloidal chitin		**Glycol chitin**					
	Days of incubation time							
	5	**15**	**5**	**15**	**5**	**15**	**5**	**15**
1. Standard strains No. 5765	6.2	13.5	7.6	16.9	0.6	10.9	462.1	414.7
2. 2nd passage of the isolate cultured on FMAY	20.3	22.7	24.4	23.1	343	3.3	454.3	688.1
3. 12th passage of the isolate cultured on SMAY	29.3	24.9	34.6	25.4	12.3	12.8	526.2	573.7

Note Standard curves of all cuticle-degrading enzymes, proteases, chitinases and lipases were shown in appendix.

CHAPTER V

DISCUSSION

One of the initial steps in the development of an entomopathogenic fungus for biocontrol is the appropriate stain selection. Virulence against the target pest is a key criterion for the developing of biocontrol agent. Therefore, the screening process was done through its pathogenicity and virulence. A fungal isolate recovered from a heavy infested insect cadaver collected in Mae Chaem district, Chiang Mai province was selected for this study by based on the previous mentioned criteria. The preliminary data was demonstrated that it could grow rapidly on artificial medium with high virulence against caterpillar pests.

In general, the characteristics of sporulation, functional and ontogenetic (24) are used to identify the entomopathogenic fungi. Despite the fact that various molecular techniques are also employed to identify the genotype of entomopathogenic fungi, *Nomuraea rileyi,* (25) but the simple method is still based upon the morphological structure. The morphological structures of the fungal isolate were used to compare with known strains of *Nomuraea rileyi* (3). It was found that the morphological characteristics of the fungal isolate were similar to those of *N. rileyi.* The conidiophores without a vesicle were loosely arranged in single, mostly verticillated and bear the very short neck phialides. Conidia were produced by phialides in dry divergent chains. The taxonomic keys using for classification of *N. rileyi* were also used to identify the fungal isolate (26). The green conidia and the phialide shape of the fungal isolate were similar to those of the genus *Penicillium*, but the arrangement of conidia of the fungal isolate along the conidiophores differs from those of *Penicillium*. The fungal isolate also differs from members of the genus *Metarhizium* in such a way that conidia of *Metarhizium* are long cylindrical, closely compacted and produced in columns, while those of the fungal isolate are broadly ellipsoidal, have smooth wall and do not produced in columns. The stage of development especially yeast-like hyphal bodies (27) indicated that the fungal isolate was similar to *N. rileyi*. The appearance of the fungal isolate on the agar plate has cream colored, mucoid and sticky growth colonies. All characteristics possess by the fungal isolate are strongly supported that it is closely related to species *N. rileyi*.

The time required for the germination of conidia of the fungal isolate on the tegument of *S. litura* was estimated by the *in vitro* study. The germination on *S. litura* occurred during 20 to 24 h after inoculation which was slower than those of *Pseudoplusia includens* and *Anticarcia gemmatilis* whose germination time were less than 8 h (28) and 6 to 18 h, respectively (7). However, the germination on *Heliothis Zea* (8) and on *Spodopter frugiperda* (29) could not observed until 48 h. It is postulated that the compositions of larval integument may provide variable nutrients for germ tube formation resulting in the variation in the initiation of spore germination on different lepidopteran larvae. It has been shown that the germination of conidia of *N. rileyi* are induced by adding larval cuticle and yeast extract to minimal medium (30). The penetration of the cuticle is usually preceded by the formation of an appressorium that firmly attaches to the epicuticle and provides the fulcrum for the mechanical and enzymatic processes, which mediated penetration. But the appressorium formation could not detect on the larval integument of *S. litura* infected by *N. rileyi*. It was probably due to the penetrations of *N. rileyi*, which primarily based upon enzymatic processes rather than mechanical processes that was different from other entomopathogenic fungi. The detection of endocuticular cell lysis prior to the invasion of hyphae into epidermis caused by the fungal isolate was similar to those described by Getzin (31) and Mohamed (8), while Boucias (7) and Thorvilson (9) did not find any extensive lysis of the endocuticle. Once the fungal isolate was successfully penetrated into the cuticle and ramified the sub-cuticular epidermis, then the laterally branch hypha entered the circulation of blood system of insect and subsequently transformed to hyphal bodies. These hyphal bodies were not recognized by hemolymph opsonins or hemocytes (blood cells) surface receptor and were not phagocytized by circulating hemocytes (32) because of the similarity between surface components of *N. rileyi* and of insect hemocytes allowing the hyphal bodies to evade host immune response. It was demonstrated that the wall surface epitopes of hyphal bodies of *Beauveria bassiana*, *Paecilomyces fumosoroseus*, and *Paeciomyces farinosus* were immediately recognized and phagocytized by the insect granulocytes and plasmatocytes (33). Since the wall surface epitopes of hyphal bodies and of insect cells are the same (34), the discrimination between wall surface epitopes of hyphal bodies and of insect cells cannot be conducted by labeled monoclonal antibody particularly against wall surface epitopes of *N. rileyi*. Therefore, the *in vitro* hyphal bodies were employed to compare *in vivo* hyphal bodies in order to verify the hyphal bodies infecting in insect cells. The multiplication of hyphal bodies requiring blood sugar was accomplished by budding and septum formation (7) until the hyphal bodies filled up in the hemocoel and initially converted to the mycelia. The transformation from non-invasion hyphal bodies to invasion mycelia provided the best formation for emerging through the cuticle of insect host. Boucias *et al*. indicated that either the depletion of nutrients and water or the expression of specific cellular component activated the conversion of the hyphal bodies (27). All infected tissues of *S. litura* larvae colonized by *N. rileyi* were similar to those found by Mohamed *et al* (8). There was no evidence of invasion of brain tissue as in *Plathypena scaba* (9) since the consumption and digestion processes of insect larvae were still active as healthy larvae. The observation of multi-layered crystals on the integument of *Bombyx mori* (35) was not found on *S. litura* integument.

The conidia of *N. rileyi* have the potential to be used as biological control agent since they can sustain its infectivity after topically applying to host larvae. Nevertheless, the hyphal bodies themselves have no potential as the conidia (36). The nutritional requirement of *N. rileyi* appeared to be more fastidious than that reported in other entomopathogenic fungi (37). This requirement limits the mass production to be used as the biological control agent. Several different formulations have been applied for the mass production of *N. rileyi*. However, the familiar medium used worldwide is Sabouraud's maltose agar supplement with 1 % yeast extract (SMAY) (38). Scale-up production of *N. rileyi* was seriously limited due to high cost of SMAY ingredients and the reduction of virulence of *N. rileyi* after serially transfer of conidia. There is a need to develop an appropriate medium for the production of this fungus if this fungus is to be used as a commercial pesticide. Consequently, peptone (nitrogen sources of SMAY medium) was replaced by fish soluble extract, the by-product of canned tuna industry, to cut down the cost of production. This novel medium consisting of fish soluble extract was called FMAY. To evaluate the use of FMAY medium, the fungal isolate was cultured on both media. All characteristics, i.e., toxicity, the rate of germination of conidia, sporulation, the production of cuticle-degrading enzymes, were determined and analyzed. It was shown that *N. rileyi* lost its ability to produce yeast-like hyphal bodies after the 6th passage of serially transfer of conidia on SMAY medium. The conidia derived from the 6th to 10th passages were pathogenic against *A. gemmatalis* but could not produce conidia on insect cadaver. However, there was a reduction in pathogenicity against insect larvae after the 10th passage of serially transfer of conidia on SMAY medium where the conidia became avirulent at the 16th passage onward (10). This observation was similar to the properties of the fungal isolate grown on SMAY medium. It was found that the decrease in pathogenicity was varied according to the number of passages but the completely lost in pathogenicity of the fungal isolate was not detected in this study. The serially passage of conidia of the fungal isolate may lead to the attenuation of the fungus and resulting in the deficiency of spore production on insect cadavers. And this observation was detected when conidia were serially passed from the 8th passage onward. It is interesting to note that the reduction in pathogenicity and spore production is not detected on the fungal isolate grown on FMAY medium. The pathogenicity of conidia obtained from the 12th passage derived from SMAY medium was reverted by subculturing on FMAY medium. This reversion may be contributed by factors found in the components of fish soluble extract. These factors may be similar to those found in larval cuticles that affected the stability of pathogenicity of the fungal isolate. Several investigators reported that the pathogenicity of entomopathogenic fungi correlated with the speed of germination of conidia (39). The reason why the conidia grown on FMAY medium could sustain their pathogenicity while that grown on SMAY medium could not prolong their pathogenicity is probably due to the difference in the speed of germination of conidia on these two media. To verify this hypothesis, the germination of conidia on both FMAY and SMAY media were compared. It was found that the conidia derived from FMAY medium germinated more rapidly and produced more conidia than those derived from SMAY medium. The speed of germination of conidia on artificial medium was corresponding to that of *in vivo* study (40). It may concluded from these findings that the speed of germination of conidia grown on FMAY is more rapid than conidia grown on SMAY. Thus, the speed of germination of conidia of the fungal isolate is contributed to the mortality rate in *S. litura* larvae.

It has been shown that the susceptible hosts of *N. rileyi* are limited to lepidoptera and two species of coleoptera because its conidia require the presence of suitable nutrient or signal transduction for regulating the germination (41). It was demonstrated that the potential of germination of *N. rileyi* depended upon the available nutrient substrates, especially the sterol, diacylglycerol and polar lipid, from the cuticular extracts that were capable to induced the germination of conidia (37). The site of interaction between conidia and insects (epicuticle) contains a wax layer and several lipids (42). Lipid associated with insect cuticle may plays important roles in the stimulation and development of germ tubes of *N. rileyi*. Since little information on the ability of lipid to the conidial germination is known, it is difficult to indicate that what kinds of lipid involved in the germination of conidia. It has been indicated that the development of conidia of the entomopathogenic fungi, *Erynia variabilis*, is regulated by fatty acids (43). Several studies of filamentous fungi indicated that fatty acids such as linoleic acid (18:2), palmitic acid (16:1) and oleic acid (18:1) were served as potential sporogenic factors in fungal development (44), (45). These fatty acids also correspond to the major fatty acids in all developmental stages of *N. rileyi*

(46). It was presumably that the fatty acids affected the morphological and physiological development of conidia. Surprisingly, the major fatty acids in fish soluble extract contain these fatty acids. This may be a reason why FMAY can retain the pathogenicity and spore production of *N. rileyi.*

The expression of proteins produced during the germ tube stage and late hyphal body stage was investigated using SDS-PAGE. It has been demonstrated that conidia of *N. rileyi* during the germ tube stage takes approximately 2 to 4 days to convert themselves into hyphal body stage (27). Therefore, the detection of protein content during germination should be performed within 3 to 4 days after inoculation. Since there were no protein patterns observed at days 3 and 4, but they were detected at day 5. The detection of protein patterns generated by mycelia was also performed at day 15 because the mycelia were mostly observed in submerged cultivation. In this study, the 2nd passage of conidia grown on FMAY medium represented as a pathogenic strain, while the 12th passage of conidia grown on SMAY medium represented as an attenuated strain. It was found that the 12th passage of conidia derived from SMAY medium was still pathogenic to *S. litura* larvae with LC_{50} value was approximately thousand times of conidia grown on FMAY medium. The culture that grown on SMAY medium failed to produce conidia. Thus, this culture was used as the attenuated strain in this experiment. The results exhibited that the patterns of protein between the pathogenic and attenuated strain were different in which the protein pattern of pathogenic strain showed two distinctive bands. The molecular weights of these bands could be estimated by mobility comparison with the known molecular weight marker on SDS-PAGE. Although the molecular weights of these bands were known but the protein structures were not determined. The protein patterns detected at day 15 of the paathogenic strain were not different from those of attenuated strain while its protein expression were less than that of attenuated strain at day 5. These results suggested that the role of proteins expressing during the germination of conidia involved in the pathogenicity of *N. rileyi*.

Attempts have been made to investigate the penetration of fungus on insect integument and results suggest that the penetration of conidia is facilitated by a combination of mechanical force and enzymatic degradation (47). But the penetration of conidia of *N. rileyi* without appressorium formation is mainly relied on enzymatic degradation. Studies are initially placed emphasis on enzymes expressed by mycelia but recently switch to focus on enzymes specifically expressed by germinating conidia of entomopathogenic fungi (48). Most entomopathogenic fungi penetrate cuticle with the aid of extracellular cuticle-degrading enzymes including proteases, chitinases and lipases (49). However, less information is known concerning the biochemical events of these enzymes responsible for the penetration and infection of *N. rileyi*. Most of evidences arose from other entomopathogenic fungi, for example the proteases produced by the entomopathogenic fungi, *Beauveria bassiana* and *Metarhizium anisopliae*, are factors related to the degree of pathogenicity of the fungus. This observation is supported by the mutants (50) which are deficient in protease production or the inhibition of protease activity (51), resulted in the reduction of the virulence against insect. It has been shown that the expression of proteases and chitinases of *N. rileyi* may correlate with the penetration of the larval integument because they expressed in greater quantities on host substrate than non-host substrate. While the expression of lipase is not significantly different on host and non-host substrate (52). The evidence that chitinases are implicated in infectivity of *N.*

rileyi coming from the comparison of chitinases production between virulent and avirulent isolates. It was found that high level of chitinases was detected in the virulent isolate but not in the avirulent isolate of *N. rileyi* (53). In this studies, the expression of protease apparently involved in the virulence of *N. rileyi* because the protease production in virulent strain was higher than that in attenuated strain at day 5, whereas the protease production at day 15 could not be detected in virulent and attenuated strain. As the results, protease enzymes presumably involve in the virulence of *N. rileyi* because they were produced during the germination of conidia. The expression of lipases and chitinases were not significantly different in both strains. It showed that these enzymes might doubtfully involved in the virulence of *N. rileyi*. However, they were necessary for the fungal growth because they were detected in all three samples. El-sayed also reported that lipases were rarely expressed by *N. rileyi* (52). Surprisingly, they were considerably generated in this study indicating that this fungus required lipids for growth rather than other nutrients. These lipids were probably used for germination of conidia as previously described in the role of lipid to the germination of conidia.

CHAPTER VI

CONCLUSION

A fungal isolate was recovered from a heavy infested cadaver of caterpillar pests collected from Mae Chaem district, Chiang Mai province located in the northern part of Thailand. The isolate was identified as entomopathogenic fungus, *Nomuraea rileyi,* by based on morphological and physiological characteristics. The pathogenicity of this isolate was studied against insect larvae, *Spodoptera litura,* in order to evaluate the possibility of using this fungal isolate as a biological control agent. The growth and development of *N. rileyi* was studied both *in vitro* and *in vivo* experiments. The growth and development of the fungal isolate was carried out through the examination of thin sections of infected larvae under the light microscope. It was shown that the infection process of *N. rileyi* began with the attachment of conidia to larval integument by non-specific hydrophobic forces. The conidia initiated invasion by germ tube was observed in 48 h. post-inoculation. It has been shown that the virulence of *N. rileyi* is depended upon the rapid germination of conidia. These are influenced by the nutrient availability on the larvae culticle such as proteins and lipids. Once the fungus was successfully penetrated into the cuticle and ramified the sub-cuticular epidermis, these invasion hyphae were proceeded to enter the circulation of blood system of insect and subsequently transformed into hyphal bodies within approximately 2.5 to 3 days. These hyphal bodies started replicating by budding and become vegetative hyphae in the hemolymph. It was found that noninvasion-hyphal bodies converted into invasion hyphae within 5.5 to 6 days after they filled up the haemocoel. These hyphal bodies were eventually transported throughout the haemocoel and gave rise to localized concentrations of mycelia in all tissues of infected larvae. Since there was no evidence of invasion of nervous tissue, the consumption and digestion processes of insect larvae were still as healthy insect. A heavy growth of intertwining mycelia developed in the haemocoel approximately 6 – 6.5 days post-exposure. Death generally occurred about 1 - 2 days later. The conidiophores emerged from insect cadaver were observed at 7 days after inoculation and subsequently cadavers became completely covered by green conidia. The complete development cycle of *N. rileyi*, in *S. litura* larvae lasted approximately 8 to 9 days.

It was observed that the conidia of the fungal isolate derived from a serially passage on SMAY medium loss its ability to produce yeast-like hyphal bodies after the 4th passage and the reduction of virulence was also observed at the same passage. The deficiency of sporulation of these conidia on insect cadavers was observed after the 8th passage onward of a serially transfer experiment as oppose to those conidia derived from a serially transfer experiment on FMAY medium that could sustain their virulence and spore production after the 8th passage. The difference in toxicity among those conidia may be contributed by the speed of germination on different media. The conidia that derived from the fungal isolate grown on FMAY medium could germinate more rapidly than those grown on SMAY. To verify this observation, the fatty acids profile of fish soluble extract used in the medium was analyzed. It was shown that there were several fatty acids found in fish soluble extract which corresponded to the lipid associated with insect cuticle that were capable of inducing the germination of conidia. These fatty acids were also found in all stage of development of *N. rileyi*. Thus, it is postulated that these fatty acids may stimulate the germination of and production of spores of *N. rileyi*. The conidia derived from FMAY medium capable of sustaining the speed of germination in every passage of a serially transfer experiment. The expression of proteins during germination of conidia plays a minor role in the virulence of *N. rileyi* because the distinct protein patterns were only observed in the virulent and attenuated strains of *N. rileyi* at day 5 on SDS-PAGE. However, the protein patterns of the virulent strain at day 15 were not different from those of attenuated strain. The molecular weights of two distinctive bands were estimated but the structure of proteins was still unknown. Several investigators proposed that cuticle-degrading enzymes released during the early period of invasion facilitated the penetration of insect cuticle by fungus. The contribution of these extracellular cuticle-degrading enzymes to virulence of *N. rileyi* could not even be certified in this study but the protease may probably involve in the penetration of larval integuments.

The local fungal isolate, *N. rileyi,* that was recovered from a heavy infested cadaver of caterpillar pests collected in Thailand was shown to be a promising agent for biological control of *S. litura*. Further studies on cuticle-degrading enzymes should be carried out by molecular techniques, for example, the comparison between wild type and mutant that is deficient in cuticle-degrading enzymes production. Those studies should reveal the effects of cuticle-degrading enzymes to the virulence of *N.rileyi*.

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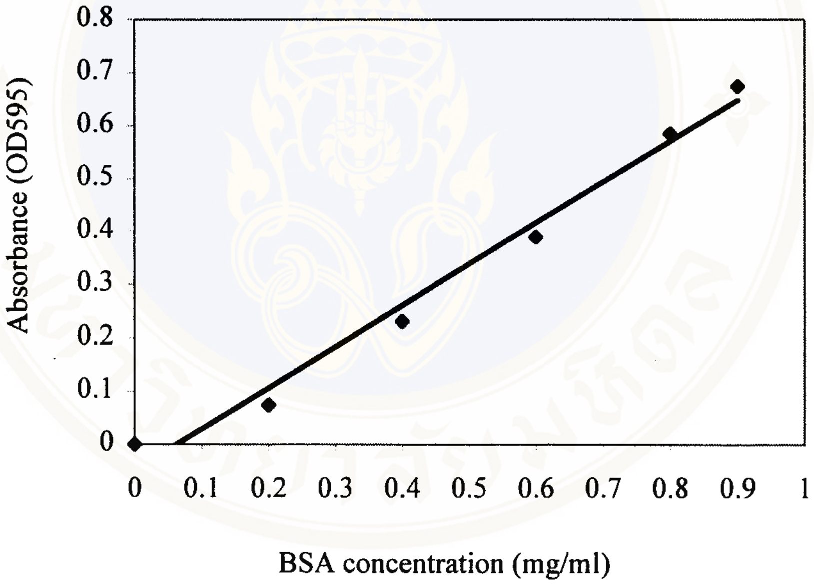
APPENDIX

Standard curves:

I. Standard curve of protein concentration determination using bovine serum albumin (BSA) measured by spectrophotometer at 595 nm.



Concentration of protein can be calculated by a following formula:

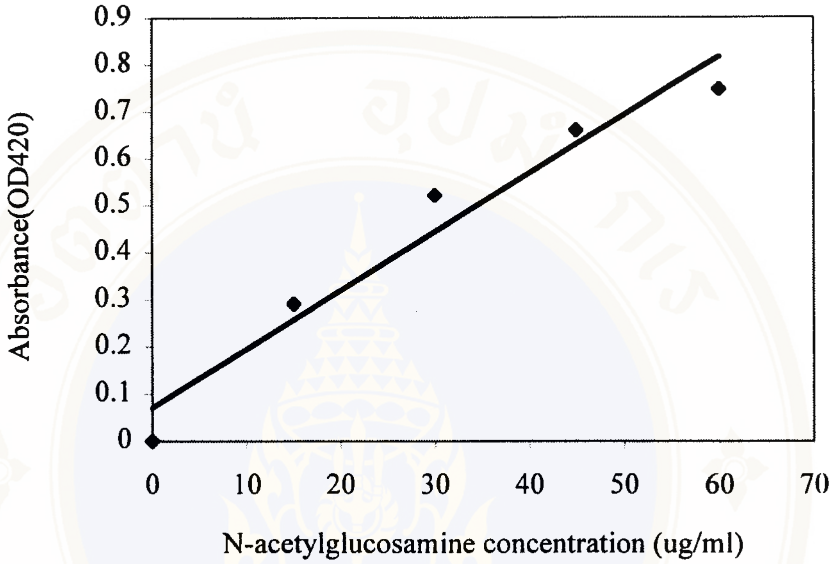
$Y = 0.7743X - 0.0489$

Y = OD value at wavelength (λ) 595

X = protein concentration (mg/ml)

$R^2 = 0.984$

II. Standard curve of N-acetylglucosamine concentration measured by spectrophotometer at 420 nm.



Concentration of N-acetylglucosamine can be calculated by a following formula:

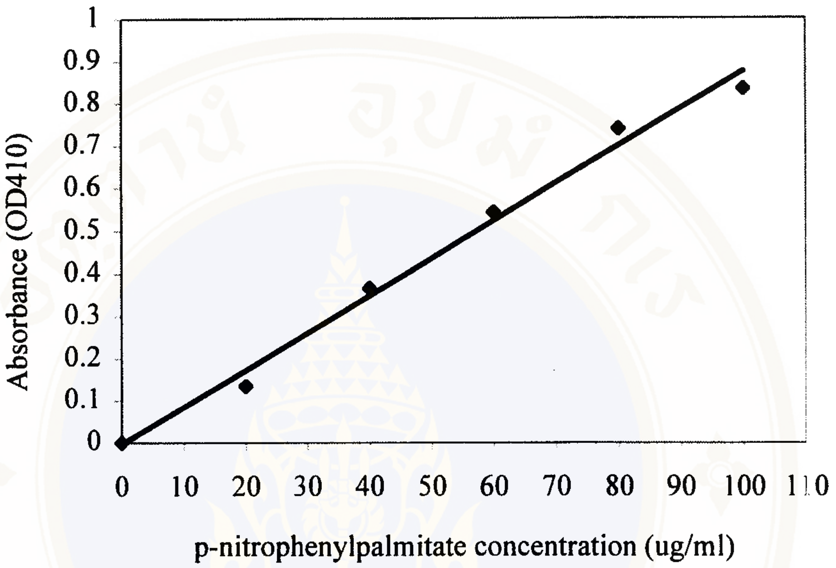
$Y = 0.0124X + 0.0713$

Y = OD value at wavelength (λ) 420

X = N-acetylglucosamine concentration (μg/ml)

$R^2 = 0.95$

III. Standard curve of p-nitrophenylpalmitate concentration measured by spectrophotometer at 410 nm.



Concentration of p-nitrophenylpalmitate can be calculated by a following formula:

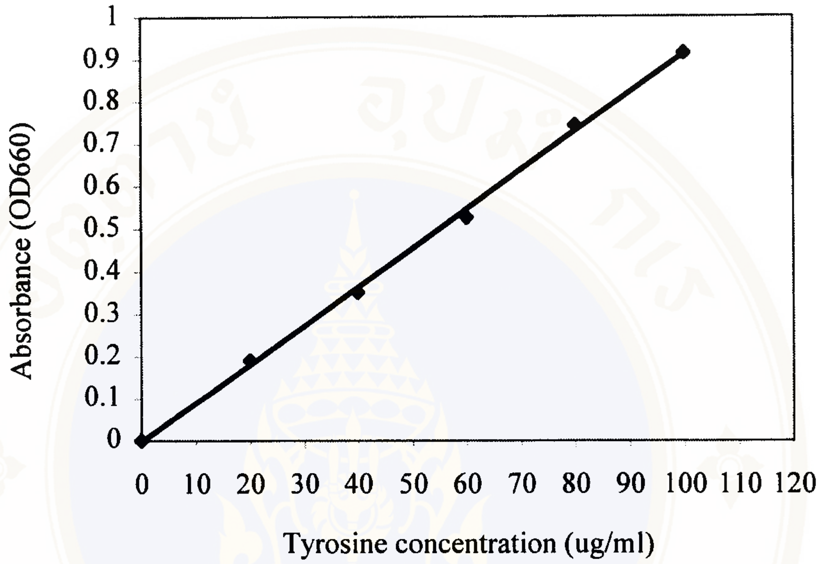
$Y = 0.0088X + 0.0037$

Y = OD value at wavelength (λ) 410

X = p-nitrophenylpalmitate concentration (μg/ml)

$R^2 = 0.99$

IV. Standard curve of tyrosine concentration measured by spectrophotometer at 660 nm.



Concentration of tyrosine can be calculated by a following formula:

$Y = 0.0091X + 0.0029$

Y = OD value at wavelength (λ) 660

X = tyrosine concentration (μg/ml)

$R^2 = 0.998$

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