BIOLOGICAL CHARACTERISTICS OF A BAMBOO FUNGUS, *Shiraia bambusicola*, AND SCREENING FOR HYPOCRELLIN HIGH-YIELDING ISOLATES

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คุณสมบัติทางชีววิทยาเชื้อราไผ่ *Shiraia bambusicola* และการคัดหาไอโซเลต
ที่ให้ผลผลิตไฮโปเครลลินสูง

นางสาวยงเฉียง หลิว

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HIGH-YIELDING ISOLATES

Suranaree University of Technology has approved this thesis submitted in fulfillment of the requirements for the Degree of Doctor of Philosophy.

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ยงเฉียง หลิว : คุณสมบัติทางชีววิทยาเชื้อราไผ่ *Shiraia bambusicola* และการคัดหาไอโซเลต
ที่ให้ผลผลิตไฮโปเครลลินสูง (BIOLOGICAL CHARACTERISTICS OF A BAMBOO
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เชื้อรา ชิราเอีย แบมบูซิโคล่า (*Shiraia bambusicola*) เป็นแหล่งของสาร ไฮโปเครลลินตาม
ธรรมชาติที่สำคัญ ความรู้ทางด้านชีววิทยาของเชื้อนับเป็นพื้นฐานสำคัญที่จะช่วยอนุรักษ์และใช้
ประโยชน์จากเชื้อราได้อย่างมีประสิทธิภาพ การศึกษาครั้งนี้มีวัตถุประสงค์เพื่อให้ทราบถึงลักษณะ
ทางสัณฐานวิทยาของเชื้อในช่วงระหว่างพัฒนาการและความสัมพันธ์กับไผ่ วงจรชีวิต พัฒนาการ
เลี้ยงเชื้อ และคัดหาไอโซเลตที่สร้างสารไฮโปเครลลิน เอ ปริมาณมาก และสภาพของการหมักที่
เหมาะสม โดยแบ่งการทดลองเป็นสองส่วน ส่วนแรกเน้นการศึกษาทางด้านชีววิทยาของเชื้อ ซึ่ง
รวมถึงการศึกษาสัณฐานวิทยาและการเลี้ยงเชื้อ การศึกษาลักษณะทางสัณฐานวิทยากระทำโดยใช้
กล้องจุลทรรศน์และกล้องจุลทรรศน์อิเลคตรอนแบบส่องกราด ศึกษาการกระตุ้นการสร้างสปอร์
โดยใช้ชนิดอาหาร ระยะเวลาการรับแสง และการเติมองค์ประกอบของพืชลงในอาหาร และทดลอง
การเลี้ยงเชื้อโดยใช้แหล่งของเชื้อ วิธีการปลูกเชื้อ และเวลาปลูกเชื้อที่แตกต่างกันบนไผ่จำนวน 4
ชนิด ผลของการศึกษาพบว่า เชื้อราสร้าง สโทรมาทา (stromata) เฉพาะบริเวณกิ่งบนของกิ่งจากปีที่
ผ่านมา แต่ไม่สามารถระบุได้ว่าเชื้อมีความสัมพันธ์แบบปรสิตกับไผ่ โดยเชื้อราเจริญอยู่รอบช่องว่าง
ของกาบใบเฉพาะกับไผ่บางชนิดเท่านั้น ได้นำเสนอและวิจารณ์ถึงวงจรชีวิตของเชื้อ ผลของ
การศึกษายืนยันได้ว่า เชื้อสร้างถุงหุ้มสปอร์แบบ 2   ชั้น และซูโดพาราไฟซิส (pseudoparaphyses)
ดังนั้นจึงควรจัดไว้ในอันดับ พลีโอสปอราเลส (pleosporales) ไม่ใช่อันดับ โดธิดีเอเลส (dothideales)
การศึกษาการพัฒนาของเชื้อในระยะ ไม่ใช่เพศและใช้เพศ พบว่าถุงหุ้มสปอร์และแอสโคสปอร์
(ascospore) มีการพัฒนาเป็นแบบ 4   ขั้นตอน คือ ขั้นตอนการสร้าง แอสคัส ไพรมอร์เดียม (ascus
primordium)   ขั้นตอนการยืดตัวของถุงหุ้ม การสร้างแอสโคสปอร์อ่อน และการสุกแก่ของแอสโค-
สปอร์ ผลของการศึกษาพบว่า วิธีแคพพิลลารี (capillary) ที่พัฒนาขึ้นเป็นวิธีแยกเชื้อสปอร์เดี่ยวที่ดี
ที่สุด และแยกเชื้อได้ทั้งหมดจำนวน 32 ไอโซเลต โดยที่ 10 ไอโซเลตได้จากสปอร์เดี่ยว 11 ไอโซเลต
จากเนื้อเยื่อ สโทรมาทา และ 11 ไอโซเลตจากกลุ่มของสปอร์ แต่ไม่พบการสร้างสปอร์ภายใต้สภาพ
ที่ใช้ในการศึกษาทุกสภาพ การปลูกและเลี้ยงเชื้อสามารถทำได้สำเร็จเฉพาะบนไผ่ ฟิลโลสแทคีส
รูโบรมาจิเนทา (*Phyllostachys rubromaginata*) หลังจากฉีดพ่นด้วยสารแขวนลอยสปอร์ที่ยังสดใหม่
การทดลองส่วนที่สอง เพื่อคัดหาไอโซเลตที่สร้างสารไฮโปเครลลิน เอ ปริมาณมาก และสภาพการหมัก
ที่เหมาะสม โดยระยะเวลาที่เหมาะสมในการสุ่มเก็บสารไฮโปเครลลินได้มาจากการศึกษาการสร้าง
ชีวมวลและปริมาณของสารไฮโปเครลลิน เอ ของเชื้อไอโซเลตที่เป็นตัวแทน ผลของการศึกษาพบว่า

เชื้อทั้ง 32 ไอโซเลต สามารถสร้างสารไฮโปเครลลิน เอ ได้สูงสุดในช่วงวันที่ 11 ถึงวันที่ 13 ของการหมัก โดยเชื้อที่ได้จากการแยกสปอร์เดี่ยวให้ผลผลิตสารไฮโปเครลลินต่ำกว่าเชื้อที่แยกได้จากกลุ่มสปอร์หรือเนื้อเยื่อสโทรมาทามาก และเชื้อไอโซเลต GZAAS2.0629 ได้รับการคัดเลือกให้เป็นไอโซเลตที่สามารถสร้างสารไฮโปเครลลิน เอ ได้สูงสุด สภาพการหมัก ได้แก่ แหล่งคาร์บอน ไนโตรเจน การเติมน้ำสกัดจากไผ่ ระดับความเป็นกรด-ด่าง เริ่มต้น ปริมาณของหัวเชื้อ และอุณหภูมิที่ใช้หมัก ได้รับการคัดเลือกโดยใช้วิธีการทดลองแบบปัจจัยเดี่ยว จากนั้นจึงจัดเป็นตำรับทดลองแบบออโธโกนอล (orthogonal) เพื่อหาอัตราส่วนคาร์บอน/ไนโตรเจน และเกลืออนินทรีย์ ในอาหารเหลวที่ใช้สำหรับการหมัก วิเคราะห์ทางสถิติโดยใช้โปรแกรม SPSS 13.0 ผลของการทดลองพบว่าการใช้อาหารเหลวที่มีส่วนผสมของ มอลโตส 3% แอมโมเนียมไนเตรต 0.001% คอปเปอร์ซัลเฟต ($CuSO_4.5H_2O$) 0.2% โปแตสเซียมไดไฮโดรเจนฟอสเฟต 0.2% มันฝรั่ง 20% และ ผงไผ่ 1% ร่วมกับการใช้หัวเชื้อในรูปของเส้นใยบนชิ้นวุ้นขนาด 4 มม. จำนวน 4 ชิ้นในอาหารปริมาตร 250 มล. พร้อมกับการเขย่าที่ความเร็ว 120 รอบต่อนาที ที่อุณหภูมิ 26 °ซ เป็นเวลา 13 วัน เป็นสภาพการหมักที่เหมาะสมที่สุดในการผลิตสารไฮโปเครลลิน เอ โดยให้สารปริมาณ 112 มก./ล.

สาขาวิชาเทคโนโลยีการผลิตพืช   ลายมือชื่อนักศึกษา_____

ปีการศึกษา 2552   ลายมือชื่ออาจารย์ที่ปรึกษา_____

        ลายมือชื่ออาจารย์ที่ปรึกษาร่วม_____

YONGXIANG LIU : BIOLOGICAL CHARACTERISTICS OF A
BAMBOO FUNGUS, *Shiraia bambusicola*, AND SCREENING FOR
HYPOCRELLIN HIGH-YIELDING ISOLATES. THESIS ADVISOR :
SOPONE WONGKAEW, Ph.D., 96 PP.

BIOLOGICAL CHARACTERISTICS/HYPOCRELLIN/SCREENING/
Shiraia bambusicola

Shiraia bambusicola is an important natural source of hypocrellin. Knowledge of biology of the fungus is fundamental to its conservation and effective utilization. This study attempted to reveal morphological characteristics of *S. bambusicola* during its developing process and its relationship with bamboo, elucidate its life cycle and develop a practicable strategy for artificial cultivation, and obtain high hypocrellin A (HA)-producing isolates and the optimized fermentation conditions. Two parts of experiments were conducted in this study. The first part focused on biology of *S. bambusicola* including morphological characters and artificial cultivation. Observation of the fungus was carried out by light microscopy and scanning electron microscopy. Sporulation induction was tried with media, photoperiod and plant supplement. Artificial cultivation for the fungus was performed with various inoculum types, inoculation methods and inoculation time on 4 bamboo species. From the observation, the fungal stromata were found only on top stalks of the previous year branches. Parasitic relationship of the fungus with the bamboo could not be indicated from the morphological evidence. The fungus grew around the interspace of bamboo

leaf sheaths and showed tissue specificity on leaf sheath of some bamboo species. A life cycle of *S. bambusicola* was proposed and discussed. The fungus was confirmed to have bitunicate asci with pseudoparaphyses, thus should be placed in the Pleosporales order and not in the Dothideales. Asexual and sexual developments of *S. bambusicola* were observed and found 4 stages of ascus and ascospore development were described. The 4 stages consisted of ascus primordium formation, ascus elongation, young ascospore formation, and ascospore maturation. A developed capillary method was found to be the best for obtaining single-spore isolates. Thirty two isolates were obtained from the isolation including 10 single spore isolates, 11 stromatal tissue isolates, and 11 multispore isolates. No sporulation could be found in the study conditions. The fungus was successfully inoculated and cultivated on *Phyllostachys rubromarginata* bamboo after being sprayed with fresh conidium suspension. The second part of the study was on screening for high HA-producing isolates of *S. bambusicola* and optimizing the fermentation conditions. Appropriate HA sampling time and screening procedure were elucidated by selecting representative isolates to determine their mycelial biomass and HA content. The 32 isolates were found to produce HA but its content in the single spore isolates were much lower than that of the isolates from multispore and stroma tissue during the sampling time at the 11th to 13th days of fermentation. The isolate GZAAS2.0629 was screened out as the highest HA-yielding isolate. Fermentation conditions of GZAAS2.0629 including carbon source, nitrogen source, bamboo extract addition,

initial pH of fermentation broth, inoculum amount, and fermentation temperature were screened with single factor experiments. Carbon/nitrogen ratio and inorganic salts in the fermentation broth were screened with orthogonal experiments. The data were analyzed with SPSS 13.0. The best fermentation condition for HA production was identified as using the broth containing 3% maltose, 1% NH_4NO_3, 0.001% $CuSO_4 \cdot 5H_2O$, 0.2% KH_2PO_4, 20% potato and 1% bamboo powder, with inoculum amount of 4 pieces of mycelial mat (4mm diameter) per 250 ml, shaking at 120 rpm and incubation at 26°C for 13 days. Under these conditions, the HA content was 112 mg/L.

School of Crop Production Technology   Student's Signature _____

Academic Year 2009   Advisor's Signature _____

                                                  Co-advisor's Signature _____

ACKNOWLEDGEMENTS

TABLE OF CONTENTS

TABLE OF CONTENTS (Continued)

TABLE OF CONTENTS (Continued)

TABLE OF CONTENTS (Continued)

TABLE OF CONTENTS (Continued)

TABLE OF CONTENTS (Continued)

LIST OF TABLES

LIST OF TABLES (Continued)

LIST OF FIGURES

LIST OF FIGURES (Continued)

LIST OF ABBREVIATIONS

Abs	=	Absorbance
BPDA	=	Bamboo and potato dextrose agar
DAPDA	=	Double antibiotic potato dextrose agar
FAA	=	Fowells acetate agar
HA	=	Hypocrellin A
HB	=	Hypocrellin B
HC	=	Hypocrellin C
MEA	=	Malt extract agar
NUV	=	Near ultraviolet
PDA	=	Potato dextrose agar
PDT	=	Photodynamic therapy
PFA	=	Potato flakes agar
PQD	=	Perylenequinoid derivative
SEM	=	Scanning electron microscope

CHAPTER I

INTRODUCTION

1.1 Background of the Study

Shiraia bambusicola P. Hennings has been reported as a parasitic fungus on branches of several genera of bamboo, which distributes only in part of southern regions of China and Japan (Kishi et al., 1991). Its relatively large stroma is used as Chinese traditional herb. The fungus is of medicinal importance because of its metabolite, hypocrellin, which has promising application in the photodynamic therapy (PDT) for anticancer treatment (Zhang et al., 1998; Yang et al., 2001; Martin et al., 2002). However, the fungus and its biological characteristics are not sufficiently known making its applications rather limited.

The natural distribution of the fungus is very limited, natural infection ratio is lower than 20% on host bamboo, and without successful artificial cultivation so far. The fungus still emerges and perishes itself in the wild. In spite of hundreds species of bamboo distribute in China, which is called *the bamboo kingdom of the world,* host bamboo of *S. bambusicola* have been reported only in a few species. With human demand for space continuously expanding, the wild resource of *S. bambusicola* is facing inevitable reduction. The previous researches have not provided enough information to protect and properly utilize of the fungal resources. In fact, there are still many questions waiting to be answered for the fungus. Does it need a specific host? What is its life cycle? What relationship is between the fungus and its host?

Where does it obtain the nutrition? And whether it can be cultivated artificially? To observe relationship between *S. bambusicola* with its host and its morphological characteristics during its life cycle, will provide better understanding of the fungus. This could lead to its successful artificial cultivation which is needed to replace the wild depleting resources.

There are more than 10 species of chemical compounds having been isolated from *S. bambusicola,* in which hypocrellin A (HA) is the main component. HA was originally isolated from stromata of *S. bambusicola* and *Hypcrella bambusae.* With the narrow distribution, wild sources of *S. bambusicola* are gradually decreasing. Moreover, *H. bambusae* has even narrower distribution existing only in the northwestern region of Yunnan province and the southeastern region of Tibet in China (Wan and Chen, 1981; Wu et al., 1989; Estey et al., 1996). Natural source of HA is far not enough to meet its extensively potential applications.

A difference between pharmaceutical fungi and edible fungi is that the stroma is not always important for the industrial production of pharmaceutical fungi. Many species of pharmaceutical fungi of which stromata are difficult to obtain have been exploited and produced industrially by using their anamorphic mycelium as substitutes, such as *Cordyceps sinensis.* However, vegetative mycelium yields less functional components than that obtained from stroma. Hypocrellin production has the same problem. Furthermore, the cost for artificial synthesis of hypocrellin is very high. Therefore, source of hypocrellin mainly depends on wild stromata from two species of fungi, *S. bambusicola* and *H. bambusae.* Due to the limitation of the wild sources, screening for hypocrellin high yielding isolates is significant for industrial production and extending application of hypocrellin.

In order to find a way to produce HA industrially, some fungal isolates have been obtained from stromata or bamboo tissues to find hypocrellin-producing strains in genus *Shiraia* (Zhang et al., 2002; Li et al., 2003a; Chen et al., 2005a; Chen et al., 2006; Liang et al., 2008). Among those reported HA-producing isolates, the fermentation conditions were different from each other. Hence, establishment of screening procedure for high HA-producing *S. bambusicola* isolates and selection for the suitable fermentation conditions are of importance for industrial HA production.

1.2 Research Objectives

(1) To study morphological characteristics of *S. bambusicola* during its developing process and relationship with bamboo

(2) To elucidate life cycle of *S. bambusicola* and develop a practicable strategy for artificial cultivation.

(3) To obtain high HA-producing isolates of *S. bambusicola* and appropriate fermentation conditions for HA-production.

CHAPTER II

BIOLOGY OF *Shiraia bambusicola*

2.1 Abstract

Biology of *Shiraia bambusicola* is the basic knowledge for its effective utilization and protection. This study attempts to reveal morphological characteristics of *S. bambusicola* during its developing process and its relationship with bamboo, elucidate its life cycle and develop a practicable strategy for artificial cultivation. Observation of the fungus was carried out by light microscopy and SEM. Sporulation induction was tried with media, photoperiod and plant supplement. Artificial cultivation for the fungus was performed with various inoculum types, inoculation methods and inoculation time on a number of bamboo species. From observation, the fungal stromata were found only on top stalks of the old branches. Parasitic relationship of the fungus with the bamboo could not be indicated from the morphological evidence. The fungus grew around the interspace of bamboo leaf sheaths and showed the tissue specificity on leaf sheath of some bamboo species. A possible life cycle of *S. bambusicola* was proposed and discussed. The fungus was confirmed to have bitunicate asci with pseudoparaphyses thus should be placed in the Pleosporales order and not in the Dothideales. Asexual and sexual developments of *S. bambusicola* were observed and for the first time, 4 stages of asci and ascospores development were described. The 4 stages consisted of ascus primordium formation, ascus elongation, young ascospore formation and ascospore maturation. No

sporulation could be found in the study conditions. Thirty two isolates were obtained from the isolation including 10 single spore isolates, 11 stromatal tissue isolates and 11 multispore isolates. A developed capillary method was found to be the best for obtaining single-spore isolates. The fungus was successfully inoculated and cultivated on *Phyllostachys rubromarginata* bamboo after being sprayed with fresh conidium suspension. The fungus has a considerably long incubation period of at least 10 months.

2.2 Introduction

S. bambusicola is an important bamboo fungus. As one of the main Chinese traditional medicines, the fungal active component, hypocrellin, has significant application on anticancer drug, edible pigment, and biological pesticides. However, the fungus and its biological characteristics have not been sufficiently known, making its applications rather limited. Its taxonomic placement has been controversial. Natural occurrence of the fungus is generally influenced by many factors such as weather and bamboo hosts. Narrow distribution, low natural infection ratio, and without successfully artificial cultivation make the wild resource facing inevitable reduction. The previous researches have not provided enough information to protect and properly utilize the fungal resources. Therefore, it is necessary to observe relationship between *S. bambusicola* with its host and its morphological characteristics during its life cycle to provide a better understanding of the fungus. All these information are essential for the development of artificial inoculation and cultivation which will lead to the fungus better protection and utilization.

2.3   Review of the Literature

2.3.1   Bamboo Fungi

Bamboo fungi were firstly termed as 'fungorum bambusicolorum' (bambusicolous fungi) by Hino (1938). 'Bambusicolous' means 'living on bamboo'. Bambusicolous includes any fungi growing on any bamboo substrates, which include leaves, culms, branches, rhizomes and roots (Hyde et al., 2002). Based on literature search and scanning the 'Index of Fungi' (http://nt.ars-grin.gov.fungaldatabase), Hyde et al. (2002) summarized that more than 1,100 species have been described on bamboo worldwide including 630 ascomycetes, 150 basidiomycetes and 330 anamorphic fungi. As being pointed out by Hyde et al. (2002), our knowledge of bamboo fungi has been incomplete and still at the cataloguing stage. Kuai (1996) listed 190 pathogenic bambusicolous fungi in Mainland China and Taiwan. And Zhou et al. (2000) reviewed that there were 189 species in 75 genera of bamboo fungi from Mainland China and 79 species in 58 genera from Hong Kong. According to the statistics of Xu et al. (2006), there were 183 species of pathogenic fungi on bamboo in China, which included 85 species from 58 genera of ascomycetes, 48 species from 58 genera of basidiomycetes and 50 species from 29 genera of hyphomycetes. In Japan 307 species (Ascomycota 63%; Basidiomycota 10%; Anamorphic fungi 27%) on 86 bamboo taxa have been reported (Tanaka and Harada, 2004).

2.3.2   Life Style Aspects of Bambusicolous

Information on the association between fungi with bamboo is mostly incomplete. The majority of pathogenic bamboo fungi have been reported from leaves with few records from culms (Boa, 1964, 1967; Parbery, 1967). Leaf spot diseases caused by several species of *Phyllachora* are one of the most common diseases of

bamboo (Boa, 1964, 1967; Parbery, 1967; Pearce et al., 2000).

Fungi are divided into two main groups：saprophytes, which can obtain nutrition from dead organic matter; pathogens and endophytes, which live on or in living plant tissues. Pathogens are capable of growing parasitically and of living saprophytically on dead organic matter, according to circumstances, referred to as either facultative parasites or facultative saprophytes (Lucas, 1998). Those can grow and reproduce in nature only on living hosts, are called obligate parasites or biotrophs (Agerios, 1997). The bambusicolous fungi of China include both pathogens and saprophytes (Zhou et al., 2000). In general the obligate parasites include rust species of *Puccinia, Stereostratum* and *Uredo*. Some of these fungi have very narrow host range and may occur only on a single variety (Shao et a1., 1984). *Phyllachora, Fusarium, Sclerotium* species are faculative parasites on bamboo. Among all 189 bambusicolous fungi known from Mainland China, 164 fungi were pathogens, which was 86.7% of the total (Kuai, 1996). At present, the greatest diversity of bamboo endophytes occurs in Asia, as of the roughly 500 species recorded, 38% were recorded in Japan (Hyde et al., 2002).

2.3.3 Distribution of Bambusicolous in China

Bamboos are highly diverse and are distributed throughout Asia, especially in China and Japan. The high number of bamboo fungi may be attributed to the high diversity of bamboo (Hyde et al., 2002). There are about 7.2 million hectare of bamboo in China accounting to 1/3 of the total bamboo forest world wide. The main distribution regions of bamboo include Anhui, Zhejiang, Fujian, Taiwan, Jiangxi, Hubei, Hunan, Chongqing, Sichuan, Guangdong, Guangxi, Guizhou and Yunnan (Xu et al., 2006). These regions are rich of bamboo species with large bamboo forest area

and have large number of bambusicolous fungi (Xu et al., 2007). Yunnan has the richest bamboo diversity in Asia with 210 woody bamboo species in 28 genera (Xue et a1., 1995). Taiwan has about 50 bamboo species in 9 genera (Hung, 1993; Huang, 1994).

2.3.4 Host Specificity of Bambusicolous

Host-specificity infers a relationship between hosts and fungi, and has mostly been applied to plant pathogens (Lucas, 1998). The obligate parasitic fungi have a very narrow host range，such as *Puccinia graminis*，greater host specificity may be detected in the interaction of particular pathogen isolates (often described as races) and specific host lines (cultivars) (Lucas, 1998).

According to calculations of Zhou et al. (2000), 63 and 55 bambusicolous fungi have been reported on *Bambusa* and *Phyllostachys* respectively．Only 11.9% bambusicolous fungi of the total 118 fungi are the same on both bamboo genera. *Puccinia longicornis* and *P. phyllostachydis* are found on both host genera, although both fungi are obligate parasitic (Kuai, 1996), it is different from the case of *Puccinia graminis*, which has high host specificity (Lucas, 1998) even they belong to the same genus. It indicates that host specificity is quite complicated and not all obligate parasitic fungi always have strict host specificity. Moreover, it was found that certain fungi were recurrent on one host, but not apparent on the other host, even in the same location, indicating that fungi may exhibit some specificity (or are recurrent) on a particular host (Zhou and Hyde, 2001).

2.3.5 Tissue Specificity of Bambusicolous

Tissue specificity infers that particular fungus is restricted to particular host tissues or organs (Lucas, 1998). Bambusicolous fungi appear to have tissue

specificity. *Phyllachora shiraiana* can be found on *Bambusa, Phyllostachys* and other several bamboo genera but is restricted to leaves (Kuai, 1996). Only 4 of the fungi occurring on *Phyllostachys* species can live on more than one kind of host tissues. With detailed host descriptions of 113 bambusicolous fungi known from Mainland China, only 6 of them are known to occur on two types of host tissues or organs (Zhou et al., 2000).

2.3.6 Economic Importance of Bambusicolous

Most bambusicolous fungi are pathogens. *Ceratosphaeria phyllostachydis* S. Zhang causes die back of *Phyllostachys pubescens* Mazel (Kuai, 1996), and has made heavy lost of bamboo forest in China. *Stereostratum corticioides* (Berk. & Broome) Magn. has wide distribution in China and makes bamboo forest a considerable loss. A list of diseases on bamboo was provided by Xu et al. (2006). Some bambusicolous fungi are medicinal. *Engleromyces goetzii* Henn., *Hypocrella bambusae* (Berk. & Broome) Sacc. and *Shiraia bambusicola* Henn., *Polyporus mylittae* Cooke et Mass., *Ganoderma lucidum* (Curtis: Fr.) P. Kars., and *G. lipsiense* (Batch) G. F. Atk. have been used as traditional Chinese medicines to treat various human diseases and of economic importance. *Dictyophora indusiata* (Vent.) Desv., which is often associated with bamboo, is well known for its medical and edible value (Ying et al., 1987).

2.3.7 Bambusicolous *S. bambusicola*

The genus *Shiraia* was first established by Hennings (1900) and placed in family Nectriaceae of Ascomycetes, but Saccardo (1902) categorized it into the family Hypocreaceae based on its large fleshy ascocarp. Description of earlier mycologists (Hennings 1900; Teng, 1934; Yamamoto and Okada, 1966) on asci of the

fungus was unitunicate, but Amano (1980) found that the genus having bitunicate asci rather than unitunicate and suggested placement of the genus in the family Pleosporaceae (Pleosporales). Kirk et al. (2001) placed the fungus as genus *Shiraia* with family *incertae sedis* in Dothideales (Dothideomycetes) in *the Dictionary of the Fungi*, 9th Edition. Based on 18S rDNA and ITS-5.8S rDNA sequences analysis, Cheng et al. (2004) classified the genus into Pleosporales as Amano (1980) proposed but suggested it in the family Phaeosphaeriaceae. So far *S. bambusicola* is the only species in the genus *Shiraia*.

2.3.8 Morphological Characteristics of *S. bambusicola*

Wei (1979) described that stroma of *S. bambusicola* was elliptical or spindle shape with surface concave, 1.5~3.0×1.0~2.0 cm, fleshy, white and smooth at first, then become soft ligneous suberin, slight pink with cracking surface, irregular warty-like or tuber-like. Perithecia were subsphaeroidal or elliptical and spreaded under the surface of the stroma with diameter of 480~590 μm. Asci were cylindrical, 280~340×22~25 μm, linearly arranged, each containing six ascospores rarely eight ascospores). Ascospores were spindle shaped with slight sharp apexes, transverse and longitudinal septa, and becoming yellow when they were accumulated. Pycnidia were produced inside the stroma. Conidiophore was short. The conidia were ascospore-like but a little bit bigger. Normally, conidia were produced prior to ascospores. Moreover, Gu et al. (1991) observed some microstructure characteristics of the fungus by light microscopy and scanning electron microscope (SEM) and suggested the asci as unitunicate.

2.3.9 Host Plant and Ecology of *S. bambusicola*

Bamboo belongs to the Poaceae (Gramineae) family and form tribe

Bambuseae of the subfamily Bambusoideae (Dransfield and Widjaja, 1995; Moulik, 1997). There are an estimated 1,000 species of bamboo belonging to 80 genera worldwide. About 200 species are found in South-East Asia (Dransfield and Widjaja, 1995). At present, some bamboo species from five genera were reported as host plants of *S. bambusicola* in China. They were *Bambusa, Fargesia, Phyllostachys, Brachystachyum*, and *Pleioblastus* (Xue, 1995; Hui et al., 1996; Lai and Fu, 2000; Cheng et al., 2004). These bamboo genera widely distribute in China (Table 2.1). Lai and Fu (2000) suggested that genus *Brachystachyum* including species of *B. densiflorum, B. densiflorum* var.*villosum, B. albostriatum*, and *B. yixingens* were the main host of the fungus through observation in the wild for many years. According to their statistics, general parasitic ratio were 1.89% on *Brachystachyum* spp. at five survey spots, but less than 0.15% on other bamboo species.

Lai and Fu (2000) found that some factors such as temperature, rainfall and relative humidity influenced emergence and transmission of the fungus. Rainwater, as a medium for fungal spore to transmit and disperse, would increase the parasitic ratio in pure bamboo forest with high relative humidity and dark conditions. It was reported that a suitable time for growth and development of the stroma was from mid April to beginning of May because of abundant rainwater and suitable temperature. Subsequently the spores would be produced largely at the end of May to beginning of June. Fewer spores were produced during the mid April to beginning of May and mid June to beginning of July with lower parasitic ratio. No spore was produced in other months.

Table 2.1   Distribution of host bamboo genera of *S. bambusicola* in China (CEFC, 1997; Plant Database)

Genus	Area of distribution	Number of species
Bambusa	East, south and south west of China	> 60
Fargesia	Area with 1,400-3,800 meters (A.S.L)	> 80
Phyllostachys	Northeast of China, Inner mongolia, Qingdao and Xinjiang	> 50
Brachystachyum	Jiangsu, Zhejiang, Anhui, Jiangxi, Hubei and Guangdong	> 4
Pleioblastus	Countrywide	20

Parasitic status of *S. bambasicola* was varied under different ecological environments. The highest parasitic ratio was found in moist and cool conditions. In the bamboo forest, higher parasitic ratio would be at the shady slope than that at the sunny slope and half-sunny side. Moreover, a type of bamboo forest was important for the fungus parasitizing. For example, the parasitic ratio in pure forest was higher nearly 30 times more than in that of the mixed forest with other species of plant (Lai and Fu, 2000). It was probably because of the advantages of transmitting and developing of the fungus in the pure forest.

Although it seems as if the fungus could induce decline on the infected bamboo, but from observation, it was found that the bamboos were not killed by parasitism of the fungus. However, there have been no reports on how much the fungus would damage the bamboo.

2.3.10 Artificial Cultivation of *S. bambusicola*

In spite of anamorphal hyphae of the fungus could be cultured on artificial medium, the fungus could not form stroma on the media or bamboo. Wu (1993) mentioned an inoculation method on cultivation of the fungus. That was spraying the fungal mycelial suspension onto host bamboo, on which stroma of *S. bambasicola* was naturally found every year. However, there was no relevant report on the result.

2.4 Materials and Methods

2.4.1 Media Used in This Study

Three types of media for general culture, storage, isolation and sporulation induction were prepared as following formulae based on 1 liter of distilled water:

1) General media:

- Potato dextrose agar (PDA): potato 200 g, dextrose 20 g, agar 15 g.

- Bamboo and potato dextrose agar (BPDA): potato 200 g, dextrose 20 g, KH_2PO_4 2 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, bamboo powder 5 g, agar 10 g.

2) Storage medium:

- PDA: the same as above.

3) Isolation medium:

- Double antibiotic medium (DAPDA): PDA + penicillin (trace) + streptomycin (trace).

4) Media for sporulation induction:

- Fowells acetate agar (FAA): agar 20 g, sodium acetate trihydrate 5 g, pH 6.5-7.0.

- Potato flakes agar (PFA): potato flakes 20 g, agar 15 g, dextrose 10 g.

- Malt extract agar (MEA): malt extract 20 g, dextrose 20 g; agar 15 g, peptone 1 g.

Bamboo powder was made from bamboo branches of *Pleioblastus amarus*. The fresh branch was washed and dried, then ground into powder using tissue pulverizer. The bamboo powder was boiled for 30 min and filtrated, then obtained the bamboo extraction.

2.4.2 Observation of *S. bambusicola* in the Wild

Growth and development of *S. bambusicola* was observed in Zhejiang of China during May to June in 2008 and 2009 which was the period when the fungus started to form stroma in the wild. Stroma forming time, growing site, host species, habitat and temperature were recorded. Stromata in different growing stages were collected and stored in formalin/acetic acid/alcohol (FAA) fixative (containing 50% alcohol 90 ml, glacial acetic acid 5 ml and formalin 5 ml) for further microscopic observation.

2.4.3 Observation with Light Microscope

The stromata collected form *Pleioblastus amarus* were cut into thin slices by hand and put into a drop of water to observe directly or, dyed with lactophenol cotton blue solution or Hoechst 33258 for observation. These specimens were observed with Olympus BX51.

2.4.4 Observation with Scanning Electron Microscope (SEM)

The SEM samples were prepared by using amended tertiary butyl alcohol freeze-drying method of Inoué & Osatake (1988) and Gao et al. (1989). The stromata of *S. bambusicola* collected from *P. amarus* were cut into small pieces and fixed with 0.5% glutaraldehyde at 4°C for 24 h. Fixed specimens were rinsed with 0.1M phosphate buffer pH 7.2 for 15 min three times. Subsequently they were further fixed with 1% osmium tetroxide in 0.1 M phosphate buffer pH 7.2 for 1 h at room temperature. After fixation, the specimens were dehydrated through a graded series of frozen tertiary butyl alcohol for 15min each in 50%, 70%, 80%, 90%, and 95% of the tertiary butyl alcohol respectively, and for 20 min in that of 100%, then further vacuum freeze dehydrated with Hitachi ES-2030 freeze dryer. The specimens were masked by ion sputtering (Hitachi E-1010) and observed with Hitachi S-3400N SEM.

2.4.5 Isolation of *S. bambusicola*

Fresh stromata collected in 2.4.2 were surface sterilized with 75% ethanol for 30 s to 1 min, then washed with sterilized water and dried with sterilized filter paper. Subsequently the stroma was cut to pick conidia or stromal tissue and transferred directly onto DAPDA plate (containing penicillin and streptomycin) for isolation. Moreover, the conidia mass was suspended into sterilized water for single spore isolation (Wang and Wen, 1997). All the culture plates were incubated at 26°C and stored with PDA slants at 4°C.

2.4.6 Sporulation Induction of *S. bambusicola*

Ten single spore isolates obtained above were used to induce sporulation. Culture pieces cut by cock borer were used as inocula. The induction methods involved media inducing (FAA, PFA, and MEA), mycelium wounding, near

ultraviolet (NUV) exposure, plant tissue supplementation or different lighting times (Dhingra and Sinclair, 1995; Atlas, 1997; Guo et al., 2003; Wang et al., 2005). All inoculated plates were incubated at 25°C or at room temperature (day 23~26°C, night 16~19°C), and observed after 7 days, 15 days and 1 month.

2.4.7 Bamboo Materials in Artificial Culture Experiment of *S. bambusicola*

The experiment was carried out at Chishui Bamboo Park in Guizhou province of China where nearly 300 species of bamboo have been reserved. Four species of bamboo, *Pleioblastus longifimbriatus*, *Bambusa multipelx*, *Phyllostachys rubromarginata*, and *Phyllostachys heterocycla* (Moso bamboo seedlings), belonging to the reported host bamboo genera were selected as testing materials.

2.4.8 Spore Collection of *S. bambusicola*

Naturally released conidia on the stroma surface at Zhejiang province were collected in 2007 and 2008 by using sterilized forceps. The collected conidia were immersed in sterilized 20% glycerol and stored at 4°C or -20°C. With the same method, ascospores were collected in 2009.

2.4.9 Inoculum Preparations of *S. bambusicola*

1) Conidium inocula

The stored conidia were diluted with sterilized 20% glycerol or mixed in 0.01% sodium alginate solution to 10^4-10^5 conidia/ml and used as inocula. The first inoculation was done by injection of the spore suspension into leaf sheath of the testing bamboo with syringe, and by spraying of that at the second time.

2) Toothpick inocula

Cultures of 32 *S. bambusicola* isolates on BPDA were prepared in

culture bottles until the hyphal growth was abundant. Subsequently, autoclaved toothpick prepared from bamboo branches were put onto the cultures for the fungus colonization at room temperature. The colonized toothpicks were further used as inocula and inoculated by insertion into the leaf sheath of the testing bamboo.

3) Wheat inocula

Wheat grains (200g) were immersed in water for 24 h until swelled, then boiled with 10 g bamboo powder for 1 h. After mixing with 5g dextrose, the treated wheat grains were distributed into culture bottles to autoclave for 1 h. Cultures of the 32 *S. bambusicola* isolates on BPDA were respectively cut into small pieces and mixed with the autoclaved wheat grains and cultured at room temperature. The colonized wheat grains were further used as inocula and inoculated by attachment on the top stalk of the testing bamboo with adhesive tape.

2.4.10 Artificial Inoculation of *S. bambusicola* on the Testing Bamboo Species

Two inoculation times were selected at before and after the fungal growing season (March and July) in 2008. Conidia inoculum in 20% glycerol was injected into leaf sheath of the testing bamboos with a syringe about 50 points for each testing bamboo species. While that in 0.01% sodium alginate solution was sprayed onto the surface of the testing bamboos for about 2 m^2. The toothpick inocula were inserted directly into leaf sheath. And the wheat inocula were adhered onto top stalk of the branches by using adhesive tape. Both toothpick and wheat inocula of each isolate were inoculated at least 10 points on different species of the testing bamboo. After inoculation, water was sprayed twice per day to keep the site moist for one week if it was not rain. The inoculation points were observed after one month.

Any stroma grew would be observed in the wild and collected for observation in the laboratory.

In order to compare effect of storage method on activity of the fungus, the spore germination rate was further tested. The spores collected in 2.4.8 were washed for 3 times with sterilized water by short centrifuging and suspended in the sterilized water containing small amount of penicillin and streptomycin. Subsequently, the spore suspension was inoculated onto sterilized cellophane sheet in a sterilized petri dish, keeping humid with sterilized 20% glycerol, and incubated at 25°C. Germination of the spores was observed with light microscope after 12 h and the germination rate was determined by counting more than 150 spores after 24 hours.

2.5   Results and Discussion

2.5.1   Observation of *S. bambusicola* in the Wild

Four species of bamboo belonging to 3 different genera were found being hosts of *S. bambusicola*. They were *Pleioblastus amarus*, *Phyllostachys praecox*, *Brachystachyum* sp., and *Phyllostachys* sp. The fungal stromata were found only on top stalks of the previous year branches. From our observation the fungus has never been found on new shoot branches. During the fungal growing season, from May to June, the temperature were about 22 to 26°C with plenty rainfall and high relative humidity.

2.5.2   Microscopic Observation

As shown in Figure 2.1 the stromata of *S. bambusicola* were pinkish irregular tuberculate surrounding the host bamboo branches (Figure 2.1 A). Ascocarps and conidia were formed in a stroma (Figure 2.1 B). Pseudothecia with opening

ostioles after maturation were immersed in the stroma tissue near its surface. Within the pseudothecia, asci were found parallelly arranged around the pseudoparaphyses (Figure 2.1 C). Most bitunicate asci contained 6 ascospores or rarely 4 ascospores (Figure 2.1 D and E). A lot of conidia were produced near the host stalk during the enlargement of the stroma and secreted on to the stromal surface. Conidiophore was short and difficult to see (Figure 2.1 F). Spore mass was yellow. Conidia and ascospores were dictyospores with transverse and longitudinal septa. The conidia were $53{\sim}79{\times}26{\sim}31$ μm in size growing on a very short conidiophore, while the ascospores were $55{\sim}80{\times}14{\sim}25$ μm (Figure 2.1 G and H).

From morphology and development of the stroma, the fungus under the study was confirmed to be *S. bambusicola.* While some reported taxonomic characteristics of the fungus were inconsistent, our observation confirmed that it had bitunicate asci and produced pseudoparaphyse inside the ascocarp. These two are the distinctive characteristics of the Pleosporales (Alexopoulos, 1962). Therefore the fungus should be placed in the order Pleosporales as suggested by Amano (1980). The placement corresponds with 18S rDNA and ITS-5.8S rDNA sequences analysis on the order classification of *S. bambusicola* by Cheng et al. (2004).
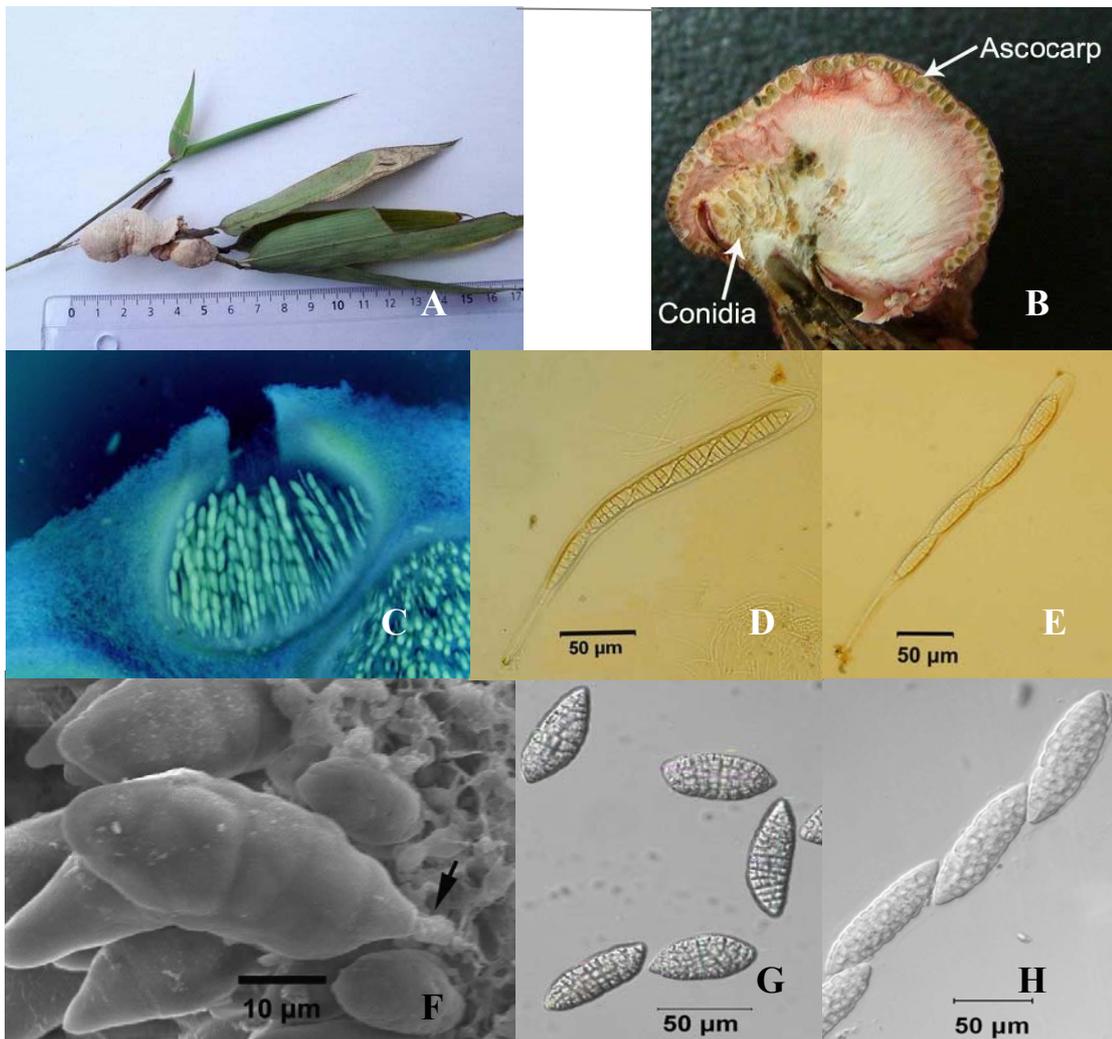
Figure 2.1 Morphology of *S. bambusicola*

A. Stroma on bamboo branch.

B. Photograph of stromal section.

C. Photomicrograph of mature pseudothecium.

D. Photomicrograph of ascus with 6 ascospores.

E. Photomicrograph of ascus with 4 ascospores.

F. SEM micrograph of conidia on a short conidiophore (arrow).

G. Photomicrograph of conidia.

H. Photomicrograph of ascospores.

2.5.3 Morphological Relationship between *S. bambusicola* and Bamboo Tissue

From our observation, most of the mycelia of *S. bambusicola* were found to grow around the interspace of bamboo leaf sheaths, intertwined with the glandular hairs but did not penetrate the plant cuticle (Figure 2.2 A and B). These mycelial mats transformed into stromal tissue among the leaf sheaths and covered the leaf sheath surface except the outermost layer (Figure 2.2 C). The fungal mycelia were found just only on the present stalk containing leaf sheath but not on stalk of the next node. Moreover, the stromal tissue could be peeled off from the host leaf sheath without damaging the cuticle (Figure 2.2 D).

The observation indicates that the fungal stromata could grow only on top branch stalks of the host bamboo. As Kuai (1996) reported the bambusicolous fungi appear to have tissue specificity．For example, *Phyllachora shiraiana* could be found on *Bambusa*，*Phyllostachys* and other several bamboo genera but was restricted to leaves. *S. bambusicola* also shows the tissue specificity on leaf sheath of several bamboo species. From the morphological evidence observed in the study, parasitic characteristics of the fungus could not be established. The host bamboo seem to show no sign of sickness (necrotic tissue or deformation), the fungal stroma could be peeled off the bamboo stalk easily, and no success in many attempts of inoculation on bamboo, all these evidence lead us to believe that the fungus may not be parasitic to the bamboo. This however does not preclude the possibility that the fungus could infect the growing stem tip hidden inside the leaf sheath. Shoot tip necrosis or die back is a very common fungal disease of many dicotyledon species because it is easily observed. This type of symptom is hardly seen in the monocotyledon species because

their growing points are obscured by the leaf sheath. Therefore to prove or disprove the parasitic relationship between *S. bambusicola* and bamboo will need more investigation.
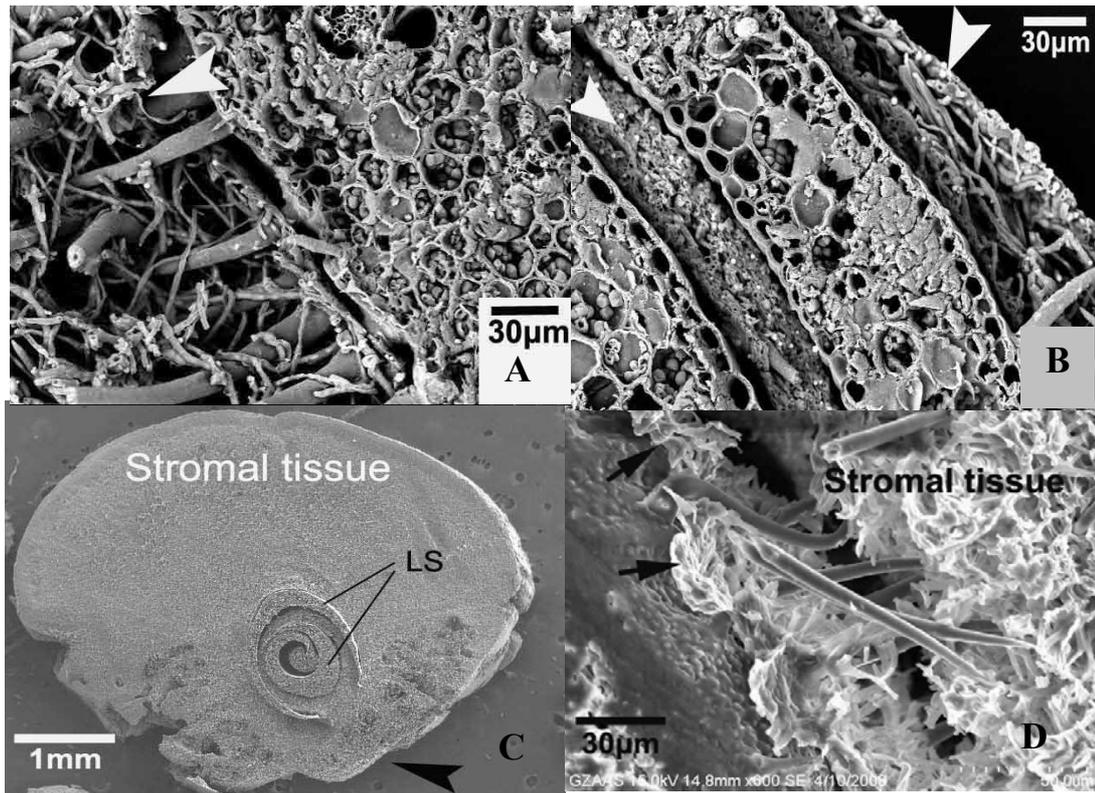
Figure 2.2 Scanning electron micrograph of transverse section of *S. bambusicola* stroma on *Pleioblastus amarus*.

    A. Mycelia intertwine glandular hairs of the leaf sheath (arrow).

    B. Mycelium mats (arrows) packed in between 2 leaf sheaths with no sign of penetrating fungal structure.

    C. Stromal tissue surrounding the leaf sheaths (LS), the outer leaf sheath (arrow) was lost.

    D. The stromal tissue on the surface of host leaf sheath (arrows).

2.5.4 Development of Asexual and Sexual Stages

As described previously, the asexual and sexual spores of *S. bambusicola* could be found together in the same stroma. When the stroma was very young, some irregular cavities had started to emerge near the side of the outermost leaf sheath, in which lots of conidia were produced continuously on the cavity wall until maturation (Figure 2.3 A). Subsequently the conidia were pushed off by the new generations of conidia and accumulated within the cavity (Figure 2.3 B). Then the mature conidia were gradually released on to the stromal surface mostly in the side of the outermost leaf sheath or partially spilled out from the stromal crack during the developing stage (Figure 2.3 C), finally leaving the empty cavities in the stroma (Figure 2.3 D).

With development of the stroma, more conidia were produced and pseudothecia began to form in the stromal tissue near surface. In our observation, there were 4 stages of asci and ascospores development. First the spherical ascus primordia emerged at the base of pseudothecium wall (Figure 2.3 E). Then the primordia grew gradually into cylindrical asci but no ascospores were found in the elongating ascus. During this period, protoplast covered by inner ascus wall also elongated simultaneously (Figure 2.3 F). The third stage was formation of the young ascospores. Transverse and longitudinal septa formed in the protoplast and figure of 6 ascospores could be observed at this stage. However, the protoplast containing 6 ascospores was not divided and kept as a whole. A special structure of apex on inner ascus wall was globose and distinct (Figure 2.3 G). The fourth stage was maturation of ascospores with transverse and longitudinal septa. At this stage, the ascospores grew bigger and become separated into individual 6 units at maturation. The special apex structure in the inner ascus wall became unclear at this period (Figure 2.3 H).

The mature ascospores finally dispersed onto stromal surface from ostiole opening of the pseudothecium (Figure 2.3 I).

Normally abundant conidia of most ascomycetes are produced for reinfection and ascospores are taken as infection source for next year. However, reinfection of *S. bambusicola* on bamboo was not found or proved in the study. The conidia formed underneath the stroma followed by forming of ascospores. The release of conidia and ascospores took place when they were mature. To a certain extent this fungus shares similarity with that of *Guignardia bidwellii* (Agrios, 1997), a pathogen of black rot of grape. It is postulated that *S. bambusicola* produces conidia and ascospores in the present year which are released and dispersed by rain water, wind or insects, then arrive and adhere to somewhere of new shooting leaf sheath of the bamboos to overwinter. When suitable conditions arrive, the conidia, ascospores or mycelia will grow in the leaf sheath and develop into new stromata to start another cycle of spore dissemination.

From our knowledge this is the first time that 4 stages of sexual development of *S. bambusicola* has been reported. Its bitunicate asci nature has also been confirmed. The unitunictate asci observed by many researchers could have resulted from the special apex structure in the inner ascus wall became unclear at ascospore maturing stage making them look unitunicate.

2.5.5 Isolates Obtained from Stromata of *S. bambusicola*

32 isoltaes were obtained from the isolation including 10 single spore isolates, 11 stromatal tissue isolates and 11 multispore isolates (Table 2.2).
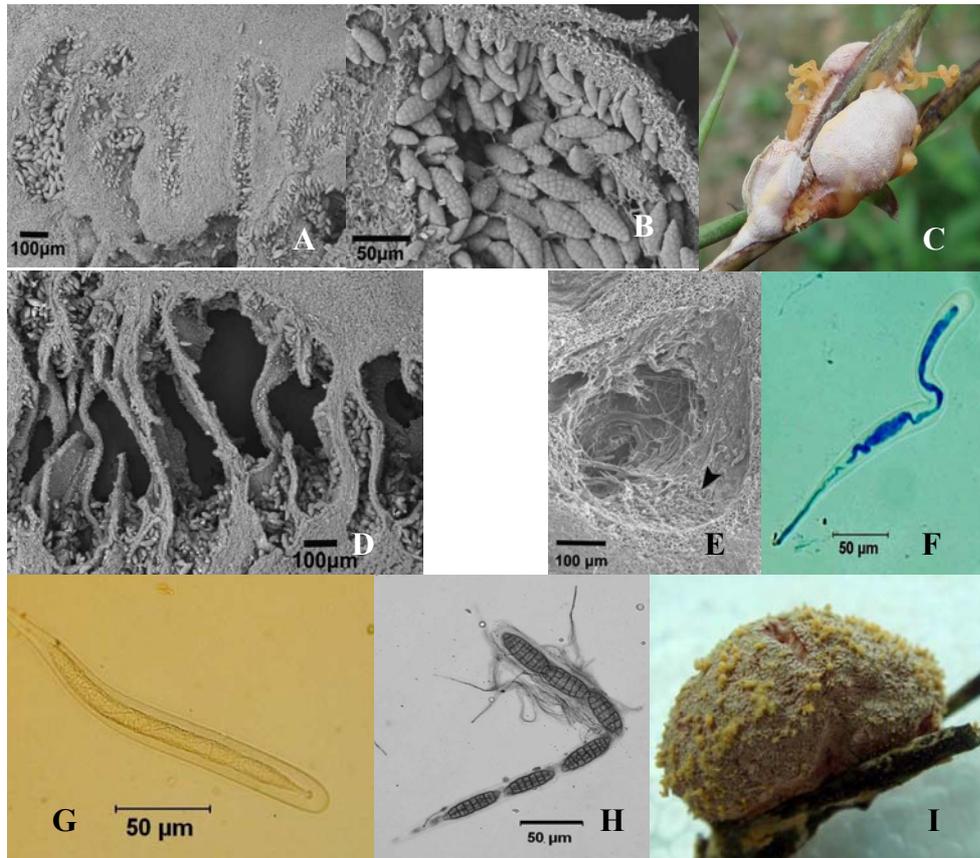
Figure 2.3 Developing process of asexual and sexual stages of *S. bambusicola* on

Pleioblastus amarus bamboo.

A. Cavities containing conidia in stroma.

B. Conidia produced on the cavity wall and mature conidia in the cavity.

C. Conidia were released onto stroma surface as yellow conidial mass.

D. Hollow cavities after conidia dispersed out.

E. Section of a pseudothecium showing ascus primordia at the base of

pseudothecium (arrow).

F. Elongating ascus.

G. Young ascospore formation.

H. Ascus with mature ascospores.

I. Mature ascospores released to stroma surface as yellow mass.

Table 2.2 Isolation result of *S. bambusicola* and relevant information.

Isolate No. *	Host	Collection site	Source
0701	*Pleioblastus* sp.	Anhui province	Single spore
0702,0703	*Phyllostachys* sp.	Linan, Zhejiang province	Single spore
0704, 0705, 0706, 0707, 0708	*Brachystachyum* sp.	Linan, Zhejiang province	Single spore
0709, 0710	*Pleioblastus* sp.	Linan, Zhejiang province	Single spore
0711	*Phyllostachys* sp.	Linan, Zhejiang province	Stromatal tissue
0612	*Pleioblastus* sp.	Panan, Zhejiang province	Stromatal tissue
0713	*Pleioblastus* sp.	Anhui province	Stromatal tissue
0714, 0715	*Phyllostachys* sp.	Linan, Zhejiang province	Stromatal tissue
0716, 0717	*Brachystachyum* sp.	Linan, Zhejiang province	Stromatal tissue
0718,0719	*Pleioblastus* sp.	Linan, Zhejiang province	Stromatal tissue
0620, 0621	*Pleioblastus* sp.	Panan, Zhejiang province	Stromatal tissue
0722	*Pleioblastus* sp.	Anhui province	Multispore
0723, 0724	*Pleioblastus* sp.	Linan, Zhejiang province	Multispore
0725, 0726	*Phyllostachys* sp.	Linan, Zhejiang province	Multispore
0727, 0728	*Brachystachyum* sp.	Linan, Zhejiang province	Multispore
0629, 0630, 0631, 0632	*Pleioblastus* sp.	Panan, Zhejiang province	Multispore

* GZAAS2. plus series number.

2.5.6   A Simple Method for Obtaining Single-Spore Isolates of *S. bambusicola*

A method was developed for obtaining single-spore isolates of *S. bambusicola* quickly with capillary (Liu et al., 2008). As is shown in Figure 2.4，that a visual field under microscope was fixed by using a section of capillary containing spore suspension to stamp on to surface of the isolation medium. The fixed visual field could be checked quickly and easily under microscope to find whether it contained one spore or not. Those medium pieces containing only one spore were further incubated at 26°C. After 1 or 2 days the pieces were transferred on to the culture medium and the isolate considered a single spore isolate.
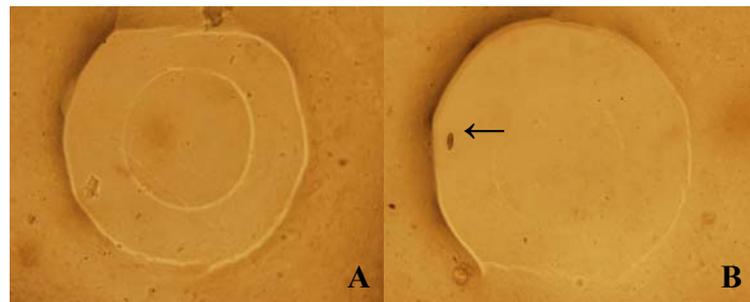
Figure 2.4   Tracing of a capillary section on the medium under a microscope at 10× objective.

A. A blank trace of capillary section on the medium.

B. A spore in the visual field of the capillary section trace on the medium (arrow).

2.5.7   Sporulation Induction of *S. bambusicola*

No spore of the fungus was found after many attempts of culturing by using different media and incubation conditions. The 10 single spore isolates (GZAAS2.0701~ 0710 in Table 2.2) had different colony morphology and characters

on each sporulation medium. After 1 month of incubation, all the isolates had very loose white mycelia growing on Fowells acetate agar (FAA) especially GZAAS2.0704 and GZAAS2.0708. Both of them could grow well producing grey mycelia on potato flakes agar (PFA) and malt extract agar (MEA). Stroma primordia could be produced easily if the colonies were physically wounded by a cork borer or transfer needle before long incubation under dry conditions. Photoperiod or NUV exposure appeared to induce the accumulation of water on the mycelial surface. And under the high humid conditions, some of the fungal hyphae transformed into sclerenchymatous cells. Other induction methods which were nutrition ladder culture, supplementation with bamboo leaf sheath, pine needle or filter paper to the fungal colonies on water agar with different pH were also failed to induce sporulation of the fungus.

Although there have been reported that *Shiraia* sp. could produce conidia easily under the lab conditions, the fungus were not confirmed as *S. bambusicola* (Liang et al., 2008). Our isolates have been confirmed as *S. bambusicola* by Liu et al. (2009, Submitted), but could not sporulate under the laboratory conditions. Therefore, artificial inoculation with the fungal spores has to depend on collection of the spores from the natural stromata in the wild.

2.5.8 Artificial Inoculation of *S. bambusicola* on the Testing Bamboo Species

Artificial inoculation was carried out in March, 2008 with different inocula and inoculation methods (Table 2.3). However, no stroma was found on the testing bamboo from April to June when the wild stroma naturally formed. The toothpick and wheat inoculum were found difficult to survive in the wild. After about

1 month of the inoculation, both toothpick and wheat inoculum became dry and nothing happened at the inoculating points.

Table 2.3 Artificial inoculation of *S. bambusicola* on 4 species of testing bamboo in March 2008 at Chishui Bamboo Park in Guizhou province of China.

Inoculum type	Inoculation method	Testing bamboo species	Inoculation result
Conidia*	Injection	*Pleioblastus longifimbriatus, Bambusa multiplx, Phyllostachys rubromarginata, Phyllostachys heterocycla*	No infection
Toothpick	Insertion	Same with above	No infection
Wheat	Adhesion	Same with above	No infection

* The conidia were collected in 2007 and stored in 20% glycerol at -20°C.

Considering fungal spores as an important factor for its natural transmission, the artificial inoculation was redone with conidia suspension spraying in July 2008. As is shown in Table 2.4, after monitoring the result of inoculation intermittently for 10 months, the fungal stromata were found to emerge only on branches of *Phyllostachys rubromarginata* sprayed with conidial suspension. The stroma could be found on the branches in and around the spraying site with sporadic distribution. The number of stoma on a bamboo plant was 1 to 10 randomly. Mature stromata of *S. bambusicola* growing on *P. rubromarginata* as a result of artificial inoculation had a size of 3~7×2~5 cm (Figure 2.5). Their relative morphological characteristics were the same as that found in the wild.

Table 2.4 Artificial inoculation of *S. bambusicola* with conidia suspension spraying on 4 species of testing bamboo in July 2008 at Chishui Bamboo Park in Guizhou province of China.

Testing bamboo species	Result
Phyllostachys rubromarginata	Stromata were found during May to June in 2009
Bambusa multiplx	No infection
Pleioblastus longifimbriatus	No infection
Phyllostachys heterocycla	No infection

Figure 2.5 *S. bambusicola* stroma resulting from artificial inoculation of *Phyllostachys rubromarginata* at Chishui Bamboo Park in Guizhou province of China.

From the result, it was the first time the fungus *S. bambusicola* was successfully cultivated on bamboo. The success of artificial cultivation is of significance for natural source protection and extensive utilization of the fungus. The artificial inoculation of *S. bambusicola* was affected by many factors such as bamboo species, inoculum type, inoculation method, inoculation time and so on. The fungus

appears to have preference for a specific bamboo species among the testing bamboos. The testing bamboo species in this study have never been reported as host of the fungus. This indicated that the fungus having host specificity even in the same bamboo genus. For many ascomycetes and basidiomycetes, stroma could be obtained from artificial cultivation on media or plant material. Mycelia on agar pieces or on plant material were usually used as inocula such as *Cordyceps minitaris* and *Wolfiporia cocos* (Li et al., 2004; Zhu et al., 2008; Qu et al., 2009). However, *S. bambusicola* has not been reported to produce stroma under the artificial inoculation or cultivation. This study has proved that mycelia on plant material could not make successful inoculation even on the right host. Considering the growing characteristics of the fungus on bamboo, without doubt the spores of *S. bambusicola* were the best inocula for artificial inoculation and cultivation on bamboo.

In order to compare effect of storage method on activity of the fungus, the spore germination test was further carried out. As shown in Figure 2.6, both ascospores and conidia of *S. bambusicola* were found germinate initially at one or two apical cells followed by others. The hypha from different cells of an ascospore could have anastomosis once they were in contact (Figure 2.6 C).

Storage temperature was the main factor to influence spore germination rate of *S. bambusicola* (Table 2.5). Both ascospores and conidia stored at 4°C for two weeks could get high germination rate of more than 90% while that of those stored at -20°C was less than 20%. Moreover, longer storage time at -20°C could adversely affect spore germination. The spores with 2 months of preservation at -20°C had 17% of germination, while that with 14 month storage had only 3% germination.
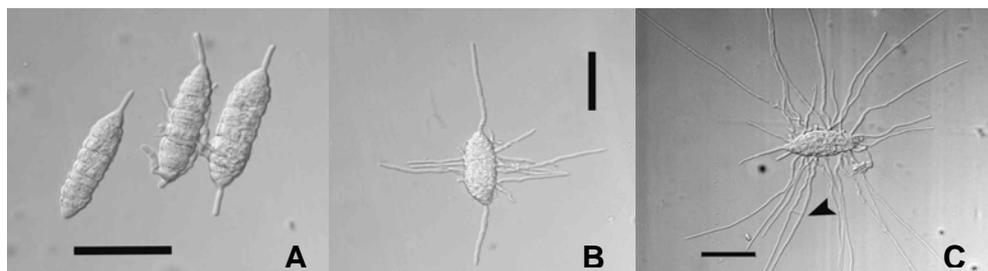
Figure 2.6 Spores germination of *S. bambusicola* on humid cellophane at room temperature. (The bar is 50 μm.)

A. Germinating ascospores.

B. Germinating conidium.

C. Anastomosis of the hypha from an ascospore (arrow).

Table 2.5 Effect of storage temperature on spores germination rate of *S. bambusicola* on humid cellophane after 24 hours at 25°C.

Sample	Spore type	Storage temperature (°C)	Storage time	Germination rate (%)
S1	Ascospores	4	2 weeks	97
A1	Conidia	4	2 weeks	93
A2	Conidia	-20	2 months	17
A3	Conidia	-20	14 months	3

In fact, a wide range of sub-zero storage temperatures are available and have been used for fungi (Smith and Onions, 1994). Storage temperature of -20°C can be used in the short-term but are inadequate for long-term storage as viability diminishes with time and properties are lost or change. For example, *Penicillium expansum* showed extensive morphological change following storage at -20°C within

the first year of storage and strains of the genus *Fusarium* showed loss in pigmentation and viability during storage at -20°C (Smith and Onions, 1994). From the result, it could explain why inoculation in March 2008 failed to cultivate *S. bambusicola* on the bamboo with spores preserved at -20°C for 10 months but succeeded for the inoculation in July with the spores preserved at 4°C. It should be the right period of inoculation for artificial cultivation before the fungal stroma occurred on the bamboo when naturally overwintered spores started germination. And injection with conidia would be a possible method of inoculation. However the spores used in the first inoculation were stored at -20°C, which made it difficult to germinate and caused failure of the inoculation before the fungal growing season. Moreover, natural performance of *S. bambusicola* in the wild also indicated influence of temperature on the stroma production. If it was a very cold winter in previous year, the stroma formed in the wild would be less than that with a warm previous winter.

According to the postulate in 2.5.4 on life cycle of the fungus, the fungus was inoculated during natural spores dissemination season by spraying the spores collected in present year on to the testing bamboos. And the fungal stromata would be obtained in the next growing season on the fit bamboo species. It is not only confirmed what we have postulated previously of the fungal life cycle, but also proved that a practicable way for artificial cultivation of *S. bambusicola* is to collect spores and help them dispersing onto the right host bamboo in the same year.

2.6 Conclusion

Stroma of *S. bambusicola* grew only on top stalks of the previous year branches without any evidence of parasitic relationship with the bamboo. It grew

around the interspace of bamboo leaf sheaths with tissue specificity on leaf sheath of several bamboo genera. The fungus had bitunicate asci and pseudoparaphyses. We suggested it should be placed in the pleosporales order. Four stages were found in development of asci and ascospore. No sporulation could be found in the study conditions. The life cycle of *S. bambusicola* was postulated and confirmed. The conidia and ascospores produced in the present year were the infection source of next year. Thirty two isoltaes were obtained from the isolation including 10 single spore isolates, 11 stromatal tissue isolates and 11 multispore isolates. A developed method was found for obtaining single-spore isolates of *S. bambusicola* quickly with capillary. It was practicable to cultivate *S. bambusicola* artificially if the active spores were inoculated onto certain host bamboo. For the first time the fungus was successfully cultivated on *Phyllostachys rubromarginata* bamboo. The fresh fungal spores produced in present year were the best inocula for artificial cultivation, but it depended on collection from the natural stromata on the bamboo.

CHAPTER III

SCREENING FOR HIGH HYPOCRELLIN A-PRODUCING ISOLATES OF *Shiraia bambusicola*

3.1 Abstract

Fungi species in genus *Shiraia* are the important source of hypocrellin produced by fermentation. The main objective of this study was to obtain high hypocrellin A (HA)-producing isolates of *Shiraia bambusicola* and the optimized fermentation conditions. Appropriate HA sampling time and screening procedure were elucidated by selecting representative isolates to determine their mycelial biomass and HA content. Thirty two isolates of *S. bambusicola* were found to produce HA but its content in the single spore isolates were much lower than that of the isolates from multispore and stroma tissue during the sampling time at the 11th to 13th days of fermentation. GZAAS2.0629 was screened out as the highest HA yielding isolate. Fermentation conditions of GZAAS2.0629 including carbon source, nitrogen source, bamboo addition and initial pH of fermentation broth, inoculum amount and fermentation temperature were screened with single factor experiments. Carbon/nitrogen ratio and inorganic salts in the fermentation broth were screened with orthogonal experiment. The data were analyzed with SPSS 13.0. The best fermentation condition for HA production was identified as using the broth containing 3% maltose, 1% NH_4NO_3, 0.001% $CuSO_4 \cdot 5H_2O$, 0.2% KH_2PO_4, 20% potato, 1% bamboo powder, with inoculum amount of 4 pieces of mycelial mat (4mm diameter)

per 50 ml, shaking at 120 rpm and incubation at 25-26°C for 13 days. Under these conditions, the HA content was 112 mg/L.

3.2 Introduction

Hypocrellin, mainly composing of hypocrellin A (HA) and hypocrellin B (HB), is a type of naturally occurring perylenequinonoid pigments with excellent photosensitive property and has promising application in the photodynamic therapy (PDT) for anticancer treatment (Zhang et al., 1998; Yang et al., 2001; Martin et al., 2002). HA and HB could be functional to kill tumour cells (Dong et al., 1987; Fu et al., 1988; Fu et al., 1989; Diwu, 1995; Fei et al., 2006; Yang and Huang, 2007), inhibit viruses and cure diabetic retinopathy (Chen et al., 2005b; Tong et al., 2004). It has been successfully employed in the clinical PDT treatment of certain skin diseases, such as white lesion of vulva, keloid, vitiligo, psoriasis, tinea capitis, and lichen amyloidosis (Xu, 1982; Liang et al., 1982; Wang and Bao, 1985; Fu et al., 1989). With the presence of visible light and oxygen, hypocrellin was reported to have a striking antiviral activity against human immunodeficiency virus type 1 (HIV-1) (Hudson et al., 1994).

HA and HB were originally isolated from stromata of *S. bambusicola* and *Hypcrella bambusae,* in which HA content was more than 95% (Xiao et al., 2003; Jiang and He, 2000). With the narrow distribution, wild sources of *S. bambusicola* and *H. bambusae* are gradually decreasing. Natural source of hypocrellin is far not enough to meet its extensive potential applications. Another way to obtain hypocrellin was extraction form anamorphic mycelium of the fungi *S. bambusicola* or *H. bambusae*. However, vegetative mycelium always yields less functional components

than that obtained from stroma. Hypocrellin production has the same problem. Furthermore, the cost for artificial synthesis of hypocrellin is very high. Therefore, screening for hypocrellin high yielding isolate is significant for industrial production and extending application of hypocrellin.

3.3 Review of the Literature

3.3.1 Metabolite Hypocrellin of *S. bambusicola*

It was reported that more than 10 species of chemical compounds had been isolated from *S. bambusicola*. They were mannitol, stearic acid, HA, HB, hypocrellin C, hypocrellin D, hypomycin A, ergosterol, ergosterol peroxide, 1,8-2 dihydroxy anthraquinone, polysaccharose, and digestive enzymes (Liu, 1987; Wang et al., 1990; Kishi et al.,1991; Lin et al., 2002b; Shen et al., 2002; Fang et al., 2006).

Hypocrellin has got widely attention in recent years owing to its excellent photosensitive properties. It was originally isolated from another bamboo fungus *Hypcrella bambusae*, (Wan and Chen, 1981) a parasitic fungus of *Sirzarurzdinaria* sp. at the northwestern region of Yunnan province in China, and was named as Shiraiachromes by some researchers because of their sources (Wu et al., 1989). To date, all of the identified perylenequinones are produced by members of the Ascomycota, the largest phylum in the fungal kingdom (Table 3.1).

3.3.2 Physical and Chemical Properties of Hypocrellin

All of the perylenequinones identified to date share a basic 3,10-dihydroxy -4,9-perylenequinone chromophore but differ mainly in side chains, such as HA and HB (Figure 3.1). It was proved that HA and HB had similar photosensitizing characteristics, generating active oxygen ($^1O_2$, $O_2^{-\bullet}$, $OH^\bullet$). The chief

target of HA and HB are the cellular membrane. Moreover, the photosensitized damage caused by hypocrellin is dependent on its form which is structurally related to perylenequinonoid pigments. Under alkaline conditions HA can be transformed to HB (Zhang et al., 1989; Zhao and Jiang, 1989; Wang and Zhang, 1992; Tian et al., 2006).

Table 3.1 Production of perylenequinones by fungi (Daub et al., 2005)

Fungal species	Ecological niche	Compound(s)
Alternaria species	Decay saprophytes on fruits and vegetables; pathogens of water hyacinth and spotted knapweed	Alteichin
A. alternate		Alterlosins
A. eichorniae		Altertoxins
		Stemphyltoxin III
Cercospora species	Plant pathogens (multiple host species)	Cercosporin
		Isocercosporin
Cladosporium cucumerinum	Saprophyte; phthogens of cucumber, sugar beet, and timothy	Calphostin C
C. cladosporidides		Cladochrome
C. herbarum		Ent-isophleichrome
C. phlei		Phleichrome
Elsinoe species	Pathogens of citrus, other plant species	Elsinochromes
Graphis hematites	Lichen	Isohypocrellin
Hypocrella bambusae	Pathogen of bamboo	Hypocrellins
Hypomyces species	Pathogen of mushrooms, shelf fungi	Hypomycin A
Scolecotrichum graminis	Pathogen of orchardgrass	Cercosporin
		Isocercosporin
		Acetylisocercosporin
Shiraia bambusicola	Pathogen of bamboo	Shiraiachromes
Stemphylium botryosum	Saprophyte; plant pathogen (multiple host species)	Stemphyltoxins
		Altertosins
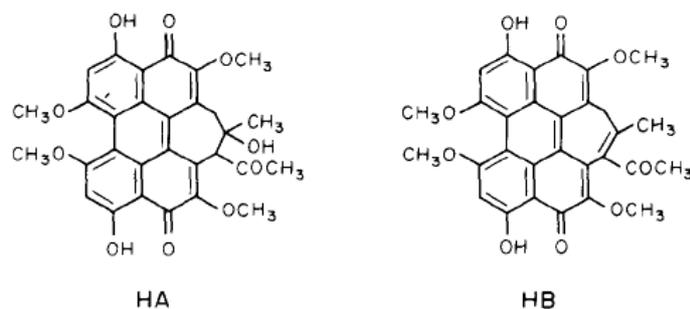
Figure 3.1 Structure of hypocrellin A (HA) and hypocrellin B (HB) (Zhang et al., 1996).

Hypocrellin dissolves in most organic solvents, such as chloroform, acetone, methanol and ethanol. It is slightly soluble in ligarine, but can not dissolve in water. Color changes from red to dark purple when hypocrellin react to iron trichloride. It is red in neutral and acidic solution, and changes from red to fresh green when alkaline solution is added. It was reported that HA in ethanol solution has three absorption peaks at 581, 542, and 463 nm respectively (Zhou et al., 2005). However, most previous research only reported that hypocrellin in ethanol solution had the absorption peaks at 465nm. HA, HB and HC have different melting point, molecular weight and spectrogram. Identification of hypocrellins could be based on their properties of solubility, color reaction and absorption peak.

3.3.3 Photosensitization Mechanism of Hypocrellin

Photosensitizers react in one of two ways. They may react by electron transfer (radical) reactions via a reducing substrate (type I reaction), leading to the production of a reduced sensitizer molecule. This molecule may react directly with cellular molecules or with oxygen, leading to the production of lipid free radicals and active oxygen species such as superoxide ($O_2^{-\bullet}$), hydrogen peroxide (H_2O_2), and the

hydroxyl radical ($OH^\cdot$) (Girotti, 1990). Alternatively, the triplet sensitizer may react directly with oxygen by an energy transfer mechanism (type II reaction) leading to the production of the non-radical, but highly toxic singlet oxygen ($^1O_2$). Almost all macromolecules in cells are susceptible to oxidative damage caused by photosensitizers. Most commonly, photosensitizers damage lipids, proteins, and DNA, with the type of damage being determined by where photosensitizers molecule localizes in cells such as in the membranes, cytoplasm or nucleus (Moan et al., 1998). Formation of activated oxygen species from photosensitizers are as shown in Figure 3.2.
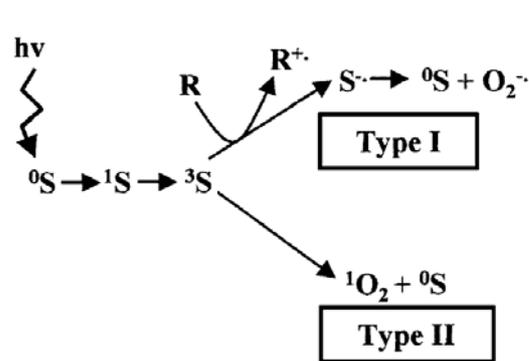
Figure 3.2   Formation of activated oxygen species from photosensitizers (Daub and
Ehrenshaft, 2000)

Photosensitizers (^0S) absorb light, and are converted to the excited singlet (^1S) and then long-lived triplet (^3S) state. The triplet sensitizer may react by an electron transfer reaction (type I reaction) through a reducing substrate (R) to generate a reduced sensitizer ($S^{-\cdot}$), which in turn reacts with oxygen to generate superoxide ($O_2^{-\cdot}$) and regenerating the ground state sensitizer. Triplet sensitizers may also react directly with oxygen via an energy transfer reaction to generate singlet oxygen ($^1O_2$) (type II reaction).

3.3.4 Application of Hypocrellin

So far, 15 patents related to hypocrellin have been issued in China, and 74 issued in the USA. Most of them related with synthesis of hypocrellin derivatives, products of hypocrellin, specific using methods, cancer or other disease treatment with hypocrellin, phototherapy, photoactivated fungicide and so on (Chinese Patent, 2007; USPTO, 2009). Moreover, hypocrellins were recommended as food additives. Because they have properties of nontoxic, tasteless, strong coloring strength, stable physical and chemical properties and health-care function (Chen, 2006). Additionally, hypocrellins were a potential novel material for optoelectronic changeover (Zhang et al., 1998; Zhang and Zhang, 1999).

HA and HB have been receiving intensive interest over the past two decades in photodynamic therapy (PDT) due to their wide absorption band in the visible region and extremely high singlet oxygen ($^1O_2$) generation ability. They have been utilized as a potent phototherapeutic agent for white lesion of vulva, keloid, vitiligo, psoriasis, tinea capitis and lichen amyloidosis (Xu, 1982; Wang and Bao, 1985). Shiraiachrome A (HA), can significantly inhibit the proliferation, migration, and tube formation of human microvascular endothelial cells (HMEC) which plays an important role in rheumatoid arthritis disease (Tong et al., 2004). This is the reason why *S. bambusicola* has long been utilized in the treatment of rheumatoid arthritis in Chinese folk medicine. Furthermore, it has been shown that hypocrellin can react against tumour cells (Dong et al., 1987; Fu et al., 1988; Fu et al., 1989; Diwu, 1995; Yang and Huang, 2007). By using human cervical cancer cells, HeLa cells, as a model, the molecular mechanisms of HA-induced tumor cell death were elucidated (Fei et al., 2006). Recent studies proved that hypocrellin has promising application in

the photodynamic therapy (PDT) for anticancer treatment (Zhang et al., 1998; Yang et al., 2001; Martin et al., 2002). Studies have also shown that hypocrellins could potently inhibit protein kinase C (Diwu et al., 1994). With the presence of visible light and oxygen, hypocrellins had a striking antiviral activity against human immunodeficiency virus type 1 (HIV-1) (Hudson et al., 1994). It was also reported that hypocrellins could photosensitize apoptosis of cells (Zhang et al., 1998; Fei et al., 2006).

Photodynamic therapy (PDT) could also be carried out on pests, such as weed and insects. The photosensitizer then was called photoactivated pesticides. Compare to common pesticides, photoactivated pesticides are a new type of harmless pesticides and have tremendous exploitable potential due to their advantages of high efficiency, high selectivity, low toxicity, low residues and safe to human and livestock. The most important components of photoactivated pesticides are photosensitizers. With the illumination and the presence of oxygen the photosensitizers act on various biochemical components and cause lethal toxic reaction in pests. The efficiency of photosensitizers depends on the quantum yield of singlet oxygen which acts on the target substrate and oxidizes it (Ma et al., 1999). Photoactivated pesticides had two sources. One was natural metabolites from plant and fungi. Another was synthetical compounds (Downum and Wen, 1995). A patent on photoactivated fungicide provided a procedure of making this fungicide by using hypocrellin as key component. The photoactivated fungicide was able to control many plant diseases, such as anthracnose of apple, cucumber grey mold, cucumber downy mildew, and phytophthora blight of pepper (Zhang and Liu, 2001).

3.3.5 Obtaining Hypocrellin from Stroma and Fermentation of Mycelium

Fungal metabolites could be obtained by extracting them from stromata or fermentation of mycelium. The former is always limited by natural sources of the fungi if they could be harvested only from the wild. Mycelial fermentation provides an excellent way to get metabolites from these fungi although there might exist some problems. Hypocrellins are in this case.

Hypocrellin is always obtained by extracting directly from stromata of *H. bambusae* and *S. bambusicola.* Chen (2006) used stromatal powder of *S. bambusicola* to extract red pigment (hypocrellin) and compared extraction efficiency in anhydrous ethanol, methanol, cyclohexane, benzene, ethyl acetate, acetone and ligarine. From his experiment, the red pigment had a similar solubility in anhydrous ethanol, methanol, benzene, ethyl acetate and acetone. When ethanol was the extract, extraction of the pigment could get the best effect if volume of the anhydrous ethanol was 50% and the ratio of solid to liquid (S/L) was 1:15. The extraction was performed at 50°C for 30 min. Moreover, the relative extraction efficiency was studied when ethanol, acetone and chloroform were used as solvents. Among them, acetone was the best one (Liu et al., 2000a). In fact, HA and HB were easily mixed during extraction process because their molecular structure were very similar. It was found that HB content was higher than HA when ethanol was used as extract. However, HA content was higher than HB if acetone was the solvent (Liu, 1996).

General procedure for getting HA was extraction and purification. Firstly, *S. bambusicola* extractum was made by using acetone. Then the extractum was washed with hot water to get rid of hydrosoluble impurities, such as mannitol. The next step was purification of HA by using column chromatography with silica gel

or Soxhlet extraction combined with acid-alkaline precipitation (Shen et al., 2002; Peng et al., 2004). The purified HA from stromata of *S. bambusicola* was 6% (Peng et al., 2004). In stroma of *H. bambusae,* HB content was very low and it was difficult to purify. Obtaining of HB depended on extraction of HA from recrystallization and further catalyzed by alkali (Tian et al., 2006).

It is known that stromata of *S. bambusicola* only exist in the wild with very limited distribution. The natural source of the fungal stromata is not enough to provide for the present demand of hypocrellin. Fermentation method could employ the fungal anamorphal mycelium to obtain hypocrellin.

Two types of fermentation method, solid and liquid state, were applied in hypocrellin production. Liu et al. (2000b) and Li et al. (2002) studied solid-state and liquid-state fermentation technology on *H. bambusae*. In the solid-state fermentation, 2% perylenequinones (PQD) was obtained when rice: corn: wheat at the ratio 2:1:1 was used as basic solid medium. Peptone at 20 g and 0.8 kg water were added to 1 kg basic medium, with unadjusted pH, and incubated at 26°C. In the liquid-state fermentation, optimized conditions could be obtained by decreasing fermentation time, improving PQD product and reducing original material.

Similarly, anamorph of *S. bambusicola* might be used to produce PQD by solid-state and liquid-state fermentation technology. However, the best conditions for HA production were quite distinct in different reports. It was found that an optimum condition in solid-state fermentation for the fungus was 1000 g rice, 50 g maltose, 20 g NH_4NO_3 and 1200 ml potato extract under unadjusted pH, and incubated for 5 days at 26°C. Under the conditions, the total perylenequinones of 1 g mycelia was about 2.4 chromo-value (Li et al., 2003a). This result was different from

another report (Cai et al., 2004), in which corn powder was the excellent basic medium and the yield of hypocrellin as 40 mg/kg. Liang et al. (2008) reported a new HA-producing strain *Shiraia* sp. SUPER-H168 with the optimum solid-state fermentation conditions as following: rice 30 g, initial pH 7.5, moisture content of 50%, incubation temperature 30°C and incubation period of 15 days. Under these conditions, the HA production could reach 2.02 mg/g dry solid substrate.

Under liquid-state fermentation, an optimized temperature for hypocrellin production was 26°C with initial pH 5.5~6. The dissolved oxygen should be abundant during the whole fermentation process. The best carbon source was glucose at concentration 30 g/L, and the nitrogen source was potato extract at 20% concentration (Shi et al., 2004). Inconsistently, Chen et al. (2005b) found that the optimum medium components and incubating conditions were: saccharose 20 g/L, yeast extract 8 g/L, corn extract 2 g/L, pH 6.0, broth quantity 50 /250 ml (V/V) flask, inoculum 10% (V/V), incubating temperature 28°C, shaking rotation 130 rpm and fermentation period 96 h. Eight grams of hypocrellin ($E_{1cm}^{1\%}465 = 52$) per liter fermentation broth was produced by using this culture condition which was exceptionally high. According to the author's description, the employed fungal anamorph was described as *S. bambusicola* but the morphological character was quite different with original description of *S. bambusicola.* Does the fungus belong to another species of fungi or is it an existing phase of *S. bambusicula* that is not found previously? This question has not been answered yet.

3.3.6 Assay Method of HA

It was reported that solution of HA in ethanol had the largest absorption in wavelength 465 nm and without interference from other components

under this wavelength. Therefore, hypocrellin could be quantified under this wavelength (Lu and Wang, 1983; Peng et al., 2004). Moreover, molecular structure of PQD compounds also supports this method of determination because PQD compounds have similar molecular frameworks and have the largest absorption at the wavelength about 465nm. Assay of HA content from fermented mycelia or stromata could be analyzed by spectrophotometry at wavelength 465 nm (Lin et al., 2002a; Shi et al., 2004).

Purification of HA could employ silica-gel thin layer chromatography (TLC) and spectrometry (Peng et al., 2004). In the former, developing solvent of TLC was chloroform/methanol (70:1, V/V), using a reference HA to compare with extracts of *S. bambusicola*. In the later, extracts of *S. bambusicola* was dissolved in acetone and made constant volume in 10ml volumetric flack. Extracts of *S. bambusicola* before and after purification were diluted to determine HA contents according to Lambert-Beer's law and extinction coefficient of HA.

3.3.7 Descriptions on HA-Producing Isolates

It was found that *S. bambusicola* isolates from different collection site with similar isolation method on diverse culture medium had distinct capabilities to produce hypocrellin (Table 3.2). Those isolates were from different laboratories and had variant morphological description, in which 3 isolates could produce conidiospore on artificial culture medium under laboratory conditions. However, morphological characteristics of those three kinds of conidia were dissimilar with each other.

Table 3.2   Differences in isolates of *S. bambusicola* used in hypocrellin production in China**.**

Collection site/ Reference	Isolation material	Culture medium for isolation	Morphological characteristics of colony	Hypocrellin produced
Wuxi, Jiangsu province (Zhang et al., 2002)	Spore	Potato extracts 16%, glucose 1.2%, bamboo leaves extracts 20% , penicillin 20 µg/L, agar 2%, natural pH, water 1000 ml	Mycelium white, light green spore produced on the top of hypha in the middle of colony after 2~3 days in 28°C, colony became slight grey during the 4th ~ 5th days and red pigment started to secrete, back of the colony became yellow to brown after 7 days, mycelium became black brown after 1month, no stroma produced.	Five isolates from 10 isolates produced hypocrellin under liquid-state fermentation.
Lin'an, Zhejiang province (Lin, 2002b)	Stromatal tissue	PDA and bamboo extracts culture medium	After incubation 5 days mycelium cotton wool-like and became black at the back of PDA plate. However, the mycelium changed from white to slight grey after 10 days and changed from red to black at the back of the plate on bamboo extracts culture medium	No hypocrellin produced.

Table 3.2   (Continued)

Collection site/ Reference	Isolation material	Culture medium for isolation	Morphological characteristics of colony	Hypocrellin produced
Lin'an, Zhejiang province (Lin, 2002b)	Spore	PDA and bamboo extracts culture medium	After incubation 7 days at 26°C, mycelium cotton wool-like and became black at the back of medium plate	No hypocrellin produced.
Leshan, Sichuan province (Li et al., 2003a)	Spore	Martin culture medium	Incubated 2 days at 26°C, mycelium white hairlike growing round radiantly, became slight red after 5days	PQD compounds and its derivative produced under solid-state fermentation.
Not given (Zhong, 2003)	Spore	Bamboo powder 20 g, glycerol 30 ml, chloromycetin 200 μg/L, maltose solution 1000 ml, agar 20 g, pH 5.5	Incubation under 27°C, early colony white hairlike, fresh red and felt-like when matured, red pigment secreted into culture medium, back of poured plate was brown red.	Hypocrellin produced under liquid-state and solid-state fermentation.

Table 3.2 (Continued)

Collection site/ Reference	Isolation material	Culture medium for isolation	Morphological characteristics of colony	Hypocrellin produced
Jiangsu province (Li et al, 2003b)	Stromatal tissue	Glucose 5g, yeast extract 5g, K_2HPO_4 1g, $MgSO_4$ 0.1g, water 1000 ml	Incubation under 26~28°C, no morphological description	Hypocrellin produced under liquid-state fermentation.
Nanjing, Jiangsu province (Chen et al., 2005a, 2006)	Not mentioned	PDA, carbon and nitrogen improved medium; corn synthetic medium	After incubation 4 days at 26°C, white aerial hypha cotton wool-like, substrate hypha became red gradually. The aerial hypha produced pycnidia 30.3-37.6×18.4-21.6 μm with unequal sphere or cucurbit-like shape, abundant conidiospore, ovate or elliptical spore with 0-1 translucent smooth septum 2.5-3.1×1.1-3.8 μm	Hypocrellin produced under both liquid-state and solid-state fermentation.

Table 3.2 (Continued)

Collection site/ Reference	Isolation material	Culture medium for isolation	Morphological characteristics of colony	Hypocrellin produced
Not given (Liang et al., 2008)	Tissue of bamboo *Barchystachyum densiflorum*	PDA	With a few white aerial mycelia after incubation at 26 for 4 days, secreted pigments made the medium turn to red. The pycnidia were much smaller about 10-20 μm in length. When the pycnidia were mature, they could release a lot of small globular or subglobular conidia with diameter less than l μm.	High hypocrellin A yield under solid-state fermentation.

3.4 Materials and Methods

3.4.1 Fungal Isolates

The isolates used in this study were the 32 isolates obtained in Chapter II as showed in Table 2.2, of which collection were in Guizhou Key Laboratory of Agricultural Biotechnology, China.

3.4.2 Culture Media

The ingredients of media were based on per liter of distilled water. All broths used in the study had 50 ml of liquid volume in a 250 ml flask. Bamboo powder preparation was the same as 2.4.1.

1) Medium for storage and determination of growth rate:

   Potato dextrose agar (PDA): potato 200 g, dextrose20 g, agar15 g.

2) Prescreening media:

   Bamboo and potato dextrose agar (BPDA): potato 200 g, dextrose 20 g, KH_2PO_4 2 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, bamboo powder 5 g, agar 10 g.

   Bamboo and potato dextrose broth (BPD broth): potato 200 g, dextrose 20 g, KH_2PO_4 2 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, bamboo powder 5 g.

3) Basal medium for optimization of fermentation conditions: BPD broth.

3.4.3 Mycelia Collection and HA Assay

Fermented mycelia were collected and washed with distilled water then dried at 60°C to constant weight. After being weighed the dry mycelia were ground into powder with dry mortar and sieved through a sieve with 60 holes per cm^2.

The mycelium powder was extracted with acetone (1g: 5ml) for 24 h. Absorbance of the extracted solution was detected with spectrophotometer at 465 nm with acetone as blank by using spectrophotometer DU 800 (BECKMAN COLTER). The HA contents was calculated based on regression equation y= 3.2x +0.02 (Shi et al., 2004).

3.4.4 Growth Rate of the Isolates

PDA cultures of the 32 isolates were cut respectively with 4 mm cork-borer, then transferred onto new PDA plates and incubated at 26°C. Growth rate of all isolates were determined everyday starting 4 days after the inoculation with 3 replications (8 points/plate). Based on statistics of the growth rate, the isolates were divided into groups.

3.4.5 Inoculum Preparation

The testing isolates were cultured on BPD agar plates (10 ml/petri dish) at 26°C for 7 days to obtain uniform colonies, which were cut into pieces with the same size as inocula by using the 4 mm cork-borer. All inocula in the next experiments were prepared in a similar way.

3.4.6 Selection of Representative Isolates and HA Sampling Time

A representative isolate was randomly selected out from each group obtained in 3.4.4 to check mycelia biomass and HA content everyday after fermentation for 4 days in the BPD broth shaking at 120 rpm at 26°C. The mycelia collection and HA content calculation of the isolates were performed with method of 3.4.3. Three HA sampling times were selected at one day before, one day after and at the peak of HA production day of each representative isolate.

3.4.7 Prescreening of HA Producing Isolates

The inocula of all isolates were prepared with the method of 3.4.5.

Each isolate was inoculated into BPD broth with three replications and cultured on a rotary shaker at 120 rpm and kept at 26°C. The mycelial collection and HA content calculation of each isolate during the sampling times were performed with method of 3.4.3. The isolates with the highest HA content were selected out for fermentation experiment.

3.4.8 Carbon Source Selection for HA Production

BPD broth without dextrose was used as contrast. Four carbon sources (dextrose, sucrose, maltose or soluble starch) with 4 levels (1%, 2%, 3%, and 4%) were respectively added into the basal broth instead of 20% dextrose. Five pieces of prepared inocula (method of 3.4.5) were inoculated into the broth. The treatments with 3 replications were cultured on a rotary shaker at 120 rpm and kept at 26°C for 13 days. The mycelial collection and HA content calculation were performed with method of 3.4.3.

3.4.9 Nitrogen Source Selection for HA Production

The best suitable carbon source and dosage tested in 3.4.8 were provided in the basal broth instead of 20% dextrose. BPD broth was used as a control. Four nitrogen sources with 3 levels, peptone (1%, 2%, and 3%), yeast extract (0.5%, 1%, and 1.5%), NH_4NO_3 (0.5%, 1%, and 1.5%), and $(NH_4)_2SO_4$ (1%, 1.5%, and 2%) were added into the broth respectively. The experiment was done in 3 replications. The inoculation, culture conditions, and HA content calculation were the same as in 3.4.8.

3.4.10 Carbon/Nitrogen Ratio Selection for HA Production

The carbon and nitrogen candidates selected in 3.4.8 and 3.4.9 were retested using L_9 (4^3) orthogonal experiment in 3 replications to look for the best

combination of C/N ratio in the fermentation medium fitting for HA production. The design was as shown in Table 3.3. The inoculation, culture conditions, and HA content calculation were the same as in 3.4.8. The best combination of C/N was tested repeatedly to compare with the BPD broth.

Table 3.3 L_9 (4^3) orthogonal design for optimization of C/N ratio.

Factors	Sucrose (%)	Maltose (%)	Peptone (%)	NH$_4$NO$_3$ (%)
Level 1	2	2	1	1
Level 2	3	3	2	2
Level 3	4	4	3	3

3.4.11 Inorganic Salt Selection for HA Production

The best carbon and nitrogen source selected in 3.4.10 were added into the basal broth instead of 20% dextrose. Two grams KH$_2$PO$_4$ and 0.5g MgSO$_4$·7H$_2$O of the basal broth were replaced by inorganic salt CuSO$_4$·5H$_2$O, KH$_2$PO$_4$, MgSO$_4$·7H$_2$O, FeSO$_4$·7H$_2$O, CaCl$_2$ or NaNO$_3$, which as 6 factors with 2 levels (Table 3.4) were tested with L$_8$ (2^6) orthogonal experiment to compare their effect on HA yield. The experiment was done in 3 replications. The inoculation, culture conditions, and HA content calculation were the same as in 3.4.8.

3.4.12 Selection of Bamboo Addition in the Medium for HA Production

Effect of different bamboo powder amount (0, 0.5%, 1%, 1.5%, and 2%) in the medium on HA yield were compared. The dextrose, KH$_2$PO$_4$, and MgSO$_4$·7H$_2$O in the basal broth were replaced by suitable carbon source, nitrogen source, and inorganic salts selected previously, in which the filtrate of each amount of

boiled bamboo powder for 30 min was added as the selection medium. The experiment was done in 3 replications. The inoculation, culture conditions, and HA content calculation were the same as in 3.4.8.

Table 3.4 L_8 (2^6) orthogonal design for optimization of inorganic salts.

Factors	$CuSO_4 \cdot 5H_2O$ (%)	KH_2PO_4 (%)	$MgSO_4 \cdot 7H_2O$ (%)	$FeSO_4 \cdot 7H_2O$ (%)	$CaCl_2$ (%)	$NaNO_3$ (%)
Level 1	0	0	0	0	0	0
Level 2	0.001	0.2	0.05	0.02	0.02	0.1

3.4.13 Initial pH Selection for HA Production

The same selection medium in 3.3.12 with original pH 5.4 was used in this experiment. Six pH gradients (5.0, 5.5, 6.0, 6.5, 7.0, and 7.5) of the selection medium were adjusted respectively by 10 M NaOH or 10 M HCl solution to compare their contributions on HA yield. The experiment was done in 3 replications. The inoculation, culture conditions, and HA content calculation were the same as in 3.4.8.

3.4.14 Selection of Inoculum Amount for HA Production

With the fermentation broth (including suitable carbon source, nitrogen source, inorganic salts and bamboo amount selected previously, potato 200 g/L, pH as result of 3.4.13), different amount of the inoculum (2, 4, 6, 8, and 10 pieces of mycelial mat) were tested for higher HA production. The inoculum preparation was the same as that in 3.4.5. The experiment was done in 3 replications. The inoculation, culture conditions, and HA content calculation were the same as in 3.4.8.

3.4.15 Selection of Fermentation Temperature for HA Production

By using the same fermentation broth as in 3.4.14, four temperature gradients (25, 27, 29, and 31°C) were tested for the maximum HA production. The experiment was done in 3 replications. The inoculation, culture conditions, and HA content calculation were the same as in 3.4.8 except the temperature of incubation.

3.4.16 Hypocrellin Production under the Improved Conditions

The repeat experiment was carried out based on previously selected conditions.

3.4.17 Statistical Analysis

The statistical analysis was done by using software SPSS 13.0 with one-way analysis of variance (ANOVA) method. Treatment means were compared using Student-Newman-Keuls test (SNK).

3.5 Results and Discussion

3.5.1 Growth Rate of the Isolates

Thirty two isolates were divided into two groups based on their growth rate (Table 3.5). Group A included 11 isolates with mean growth rate of 1 mm/day. Other 21 isolates with mean growth rate of 2 mm/day were put into group B.

Table 3.5 The *S. bambusicola* groups based on mean growth rate on PDA at 26°C.

Group	Mean growth rate (mm/day)	Isolate No. (GZAAS2. plus series number)
A	1	0702, 0703 , 0612, 0713, 0715, 0620, 0621, 0722, 0629, 0630, 0631
B	2	0701, 0704, 0705, 0706, 0707, 0708, 0709, 0710, 0711, 0714, 0716, 0717, 0718, 0719, 0723, 0724, 0725, 0726, 0727, 0728, 0632

3.5.2 Selection of Representative Isolates and HA Sampling Time

Isolates GZAAS2.0703 and GZAAS2.0707 were selected as representatives of group A (1 mm/day mean growth rate) and group B (2 mm/day mean growth rate) respectively. Growth curve of the representative isolates (Figure 3.3) showed the dynamic change of biomass and HA content. Initially, both biomass and HA content increased with time. The biomass of mycelia gradually increased from the 5th day to the 13th day then kept in a steady stage. The highest HA yields were obtained at the 12th day in both isolates. Therefore, the 11th to 13th day were selected as sampling time to screen for high HA-producing isolates.
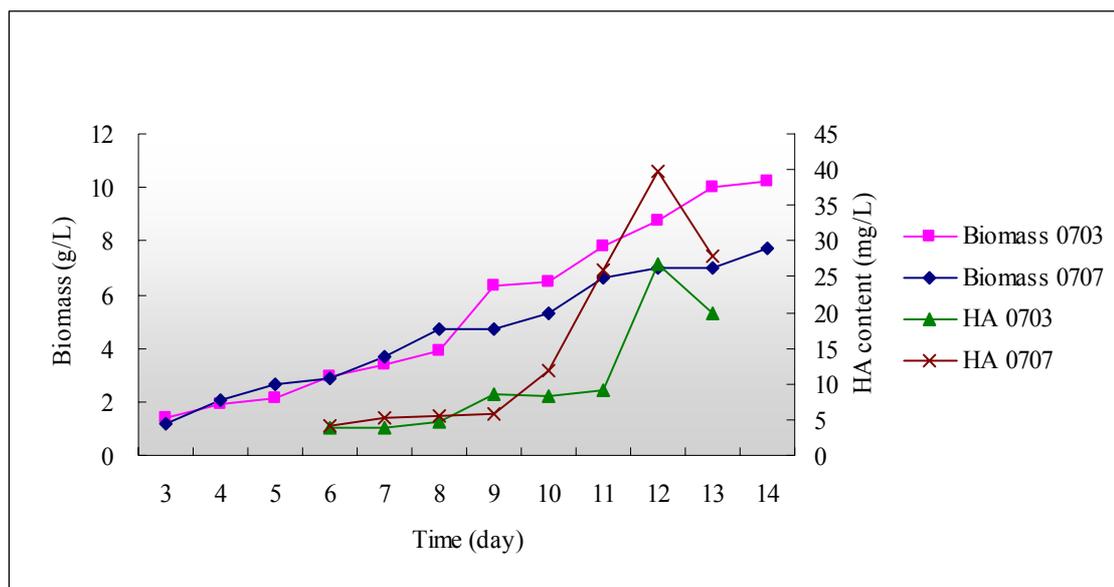
Figure 3.3 Biomass and HA producing curve of 2 *S. bambusicola* representative isolates, cultured in BPD broth and shaking at 120 rpm at 26°C.

3.5.3 Prescreening of HA Producing Isolates

All isolates in this study could produce HA during liquid fermentation (Figure 3.4). Most isolates produced the highest HA content at the 13th day. The isolate GZAAS2.0629 was found to produce the highest HA at the three sampling days among all the testing isolates. The single spore isolates, no. 1 to 10, showed uniformly lower HA contents. Thus the GZAAS2.0629 was screened out as the fermentation isolate in the next study.
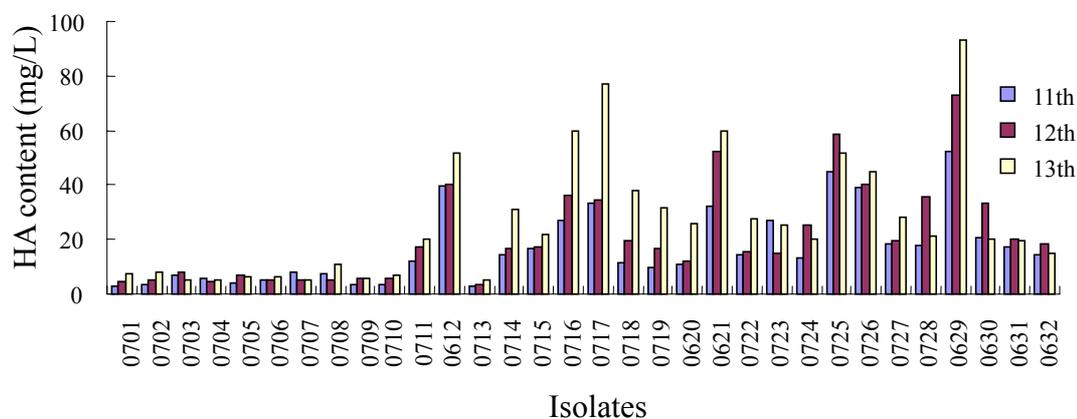
Figure 3.4   HA content during fermentation at the 11th to 13th day of *S. bambusicola*

isolates, cultured in BPD broth and shaking at 120 rpm at 26°C.

3.5.4 Carbon Source Selection for HA Production

GZAAS2.0629 isolate could grow and produce HA in the broths with

testing carbon sources dextrose, sucrose, and maltose, except in that with soluble

starch. The fungus nearly stopped growth even in the broth with 1% soluble starch. As

shown in Figure 3.5, with raise of the carbon source concentration in the broths, both

mycelium dry weight and the HA content continuously increased (Figure 3.5 A and C).

The higher HA content was obtained in the same concentration carbon sources of

maltose and sucrose than that of dextrose, but the absorbance of the extracted solution

successively rose until each carbon source concentration reached 3% then started to

decrease (Figure 3.5 B). As is shown in Table 3.6, the mycelial biomass and HA

content of treatments with maltose concentration at 3% and 4% were significantly

different from that of other treatments and the control. Therefore 3% maltose should

be the best carbon source under the testing conditions. Moreover, HA contents

obtained from sucrose (3% and 4%), dextrose (3% and 4%), and 2% maltose were

significantly different from that of other low concentration treatment.
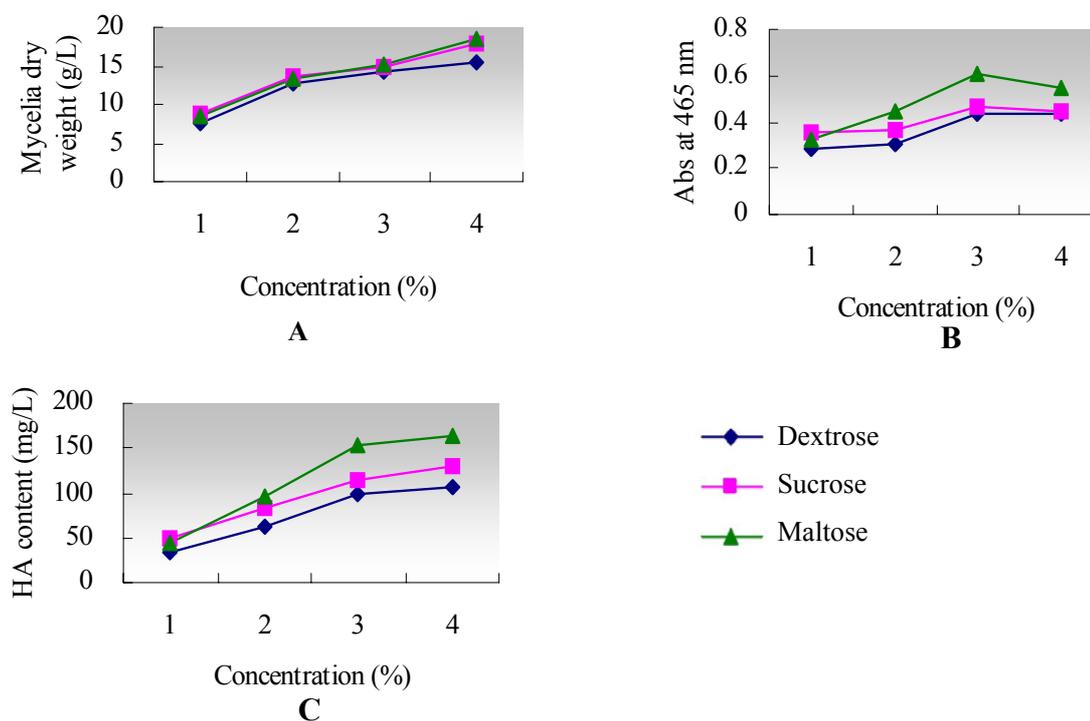
Figure 3.5 Effect of carbon sources on *S. bambusicola* GZAAS2.0629 when cultured at 26°C with 120 rpm at the 13th day.

A. Effect of carbon sources on mycelial weight.

B. Effect of carbon sources on absorbance (Abs) at 465 nm of acetone extracting solution.

C. Effect of carbon sources on HA content.

Carbon element widely exists in microorganism protoplast and its metabolites, which performs the role of energy source in the life activities. It is one of the basic nutrient elements and required largely by the microorganism (Zhou, 1996). In this study, the testing disaccharides, maltose and sucrose, were better than dextrose in promoting HA production under the testing conditions. As indicated by Shi, et al. (2004) and Lou, et al. (2006), excessive dextrose could inhibit HA production. Optimum carbon source level was found in this study which benefit the increase of

mycelial biomass, but at higher concentration they appear not to increase the HA content. The HA yield measured as the content per mycelial unit weight did not increase with the increase of carbon sugar concentration after the 3% level.

Table 3.6 Effect of carbon source on mycelial growth and HA production of *S. bambusicola* GZAAS2.0629 cultured at 26°C with 120 rpm for 13 days.

Carbon source	Mycelial dry weight (g/L)*	HA content (mg/L)*
Control (BPD broth)	3.07 ± 1.49 e	22.47 ± 9.95 e
Dextrose 1%	7.54 ± 0.38 d	34.44 ± 1.98 de
Dextrose 2%	12.60 ± 0.18 d	61.65 ± 14.75 cde
Dextrose 3%	14.13 ± 0.27 abc	99.74 ± 13.12 abcd
Dextrose 4%	15.51 ± 2.30 abc	107.46 ± 12.71 abc
Sucrose 1%	8.73 ± 2.40 d	49.31 ± 10.82 cde
Sucrose 2%	13.66 ± 0.95 bc	81.86 ± 27.09 bcde
Sucrose 3%	14.94 ± 1.22 abc	113.61 ± 7.55 abc
Sucrose 4%	17.88 ± 2.10 ab	129.95 ± 12.85 ab
Maltose 1%	8.57 ± 0.44 d	44.81 ± 4.04 cde
Maltose 2%	13.39 ± 0.57 bc	96.78 ± 11.20 abcd
Maltose 3%	15.28 ± 2.81 abc	153.78 ± 55.76 a
Maltose 4%	18.58 ± 1.22 a	164.01 ± 31.19 a

* Mean of three replications ± SD, followed by a common letter in each column are not significantly different by SNK at 1% level.

3.5.5 Nitrogen Source Selection for HA Production

In this experiment, 3% maltose selected previously was supplied instead of 2% dextrose in the basal broth. As shown in Figure 3.6, among the testing nitrogen sources, peptone showed the best for the mycelial biomass, of which both dry mycelial weight and Abs value at 465 nm had positive relationship with its concentration, but each concentration of NH_4NO_3 gave a higher absorbance than that of the other treatments (Figure 3.6 A and B). Although the HA content obtained from using 3% peptone was significantly different from that of the control, there were no differences among other treatments (Table 3.7).

Nitrogen element is the main material for synthesis of protein which is the primary component of cells for maintaining life. Generally, parasites prefer natural organic nitrogen than inorganic nitrogen (Zhou, 1996). This study also showed better utilization of the organic nitrogen (peptone and yeast extract) for mycelium growth than that of the inorganic nitrogen (NH_4NO_3 and $(NH_4)_2SO_4$) (Table 3.7). However, NH_4NO_3 and $(NH_4)_2SO_4$ had higher contribution to HA content in a unit weight of mycelia. The result is accordant with report of Li et al. (2002). They suggested that addition of cheaper $(NH_4)_2SO_4$ and reduction of expensive peptone to produce Perylenequinoid derivative (PQD) was a good way to reduce the cost, even though the total yields of PQD might be influenced to a certain extent. Moreover, $NaNO_3$ also had been reported as the best nitrogen source of fermentation for HA by Lou et al. (2006).
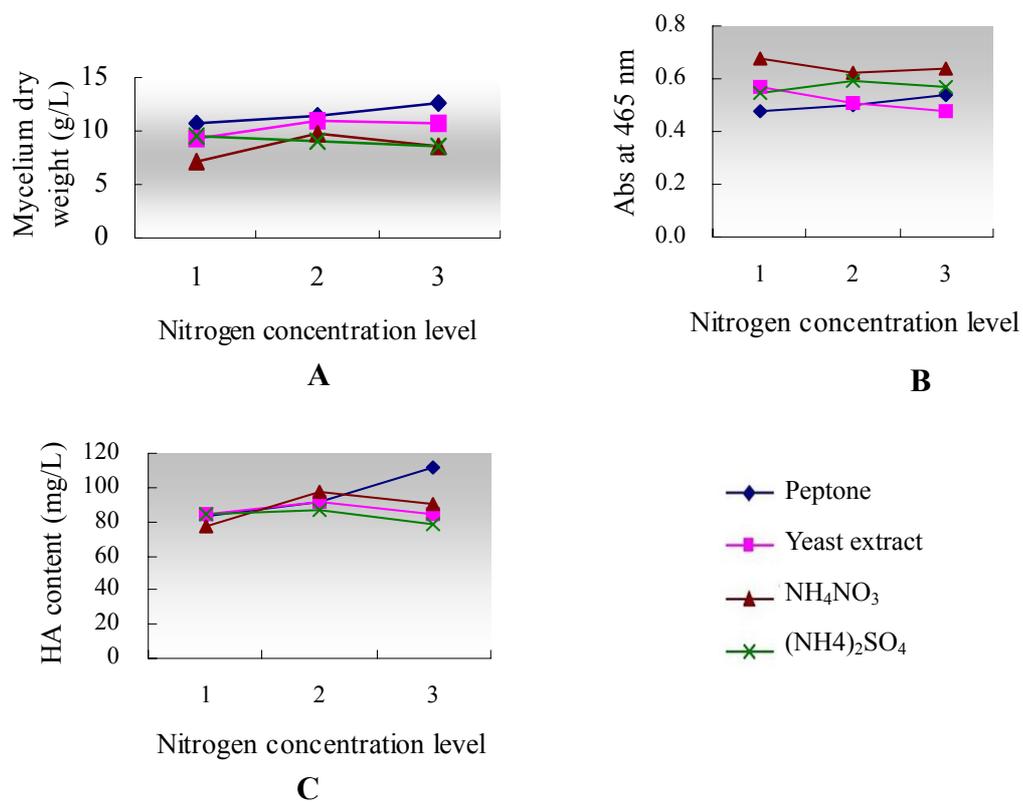
Figure 3.6 Effect of nitrogen sources on *S. bambusicola* GZAAS2.0629 fermented at 26°C with 120 rpm at the 13th day (peptone 1%, 2%, and 3%; Yeast extract or NH_4NO_3 0.5%, 1%, and 1.5; (NH4)$_2$SO$_4$ 1%, 1.5%, and 2%).

A. Effect of nitrogen sources on mycelial weight.

B. Effect of nitrogen sources on absorbance (Abs) at 465 nm of acetone extracting solution.

C. Effect of nitrogen sources on HA content.

Table 3.7 Effect of nitrogen source on mycelial growth and HA production of *S. bambusicola* GZAAS2.0629 cultured at 26°C with 120 rpm for 13 days.

Nitrogen source	Mycelial dry weight (g/L)*	HA content (mg/L)*
Peptone 1%	10.81 ± 0.23ab	83.43 ± 10.71 ab
Peptone 2%	11.41 ± 1.43ab	92.00± 6.70 ab
Peptone 3%	12.73 ± 1.53a	111.81± 32.27 a
Yeast extract 0.5%	9.18 ± 0.19bc	84.62 ± 2.12 ab
Yeast extract 1%	10.95 ± 1.36ab	91.18 ± 23.82 ab
Yeast extract 1.5%	10.74 ± 1.55ab	83.84 ± 17.66 ab
NH_4NO_3 0.5%	7.09 ± 0.50c	77.52 ± 4.02 ab
NH_4NO_3 1%	9.69 ± 1.31abc	97.47 ± 8.36 ab
NH_4NO_3 1.5%	8.67 ± 1.41bc	89.81 ± 20.16 ab
$(NH_4)_2SO_4$ 1%	9.52 ± 1.40bc	84.17 ± 9.07 ab
$(NH_4)_2SO_4$ 1.5%	9.07 ± 1.35bc	86.33 ± 18.20 ab
$(NH_4)_2SO_4$ 2%	8.67 ± 1.24bc	78.99 ± 5.85 ab
Control (BPD broth)	6.69 ± 1.23c	55.28 ± 13.53 b

* Mean of three replications ± SD, followed by a common letter are not significantly different by SNK at 5% level.

3.5.6 Carbon/Nitrogen Ratio Selection for HA Production

C/N ratio in a medium is very important for mycelial growth and production of metabolites. According to the screening results of carbon and nitrogen sources in 3.4.5 and 3.4.6, sucrose and maltose in the fermentation broth showed very good performance on increasing mycelial biomass, Abs at 465 nm and HA content. Organic nitrogen peptone and inorganic nitrogen NH_4NO_3 also displayed the stimulating effect upon them. The result of L_9 (4^3) orthogonal experiment on mycelial

biomass and HA production was shown in Table 3.8.

Table 3.8   Result of L_9 (3^4) orthogonal test on mycelial biomass and HA production of *S. bambusicola* GZAAS2.0629 cultured in varied C/N ratio media at 26°C with 120 rpm for 13 days.

Run	A*	B	C	D	Mycelial dry weight (g/L)	HA content (mg/L)
1	1	1	1	1	8.07 ± 0.30	53.11 ± 3.71
2	1	2	2	2	7.20 ± 0.69	48.81 ± 6.70
3	1	3	3	3	6.16 ± 0.68	35.28 ± 8.93
4	2	1	2	3	6.29 ± 0.36	32.35 ± 5.85
5	2	2	3	1	8.46 ± 1.33	52.57 ± 15.86
6	2	3	1	2	6.68 ± 0.72	47.72 ± 6.34
7	3	1	3	2	8.12 ± 1.51	52.82 ± 14.77
8	3	2	1	3	4.81 ± 0.14	27.82 ± 1.24
9	3	3	2	1	9.57 ± 0.25	50.60 ± 11.59

* A, B, C, and D represent factors of sucrose, maltose, peptone and NH_4NO_3 of 3 levels as indicated in Table 3.3 respectively. Data given are means of three replications ± SD.

As shown in Table 3.9, the best combination of C/N contributing to the highest mycelial dry weight was $A_3B_1C_2D_1$, which was very close to $A_3B_3C_2D_1$ as the test 9 in Table 3.8. And the best C/N for HA production was $A_1B_1C_3D_1$, namely, sucrose: maltose: peptone: NH_4NO_3 as 2: 2: 3: 1. According to the range data, R(D) > R(C) > R(B) > R(A), NH_4NO_3 had the highest contribution to the mycelial dry weight and the HA content, followed by peptone, maltose, and sucrose. However, variance analysis (Table 3.10) indicated that only NH_4NO_3 was significantly different from other factors, and 1% NH_4NO_3 was significantly different from that of 2% and 3%. Because the best combination $A_1B_1C_3D_1$ did not occur in the tested treatment

combinations, $A_1B_1C_3D_1$ and BPD broth were tested to compare their contribution for HA production in the repeated experiment.

Table 3.9 Analysis of C/N ratio on mycelial biomass and HA production of *S. bambusicola* GZAAS2.0629 with L_9 (3^4) orthogonal test.

	Mycelial dry weight (g/L)				HA content (mg/L)			
	A*	B	C	D	A	B	C	D
k 1	7.14	7.49	6.52	8.70	45.73	46.06	42.88	52.09
k 2	7.14	6.82	7.69	7.33	44.18	43.07	43.89	49.78
k 3	7.50	7.47	7.58	5.75	43.75	44.53	46.89	31.78
R	0.36	0.67	1.17	2.95	1.99	2.99	4.01	20.31

* A, B, C, and D represent factors of sucrose, maltose, peptone and NH_4NO_3, respectively.

k_i^X = Mean of the factor at each level. $R_i^X = \max k_i^X - \min k_i^X$.

X represents factor A, B, C, or D, respectively, and symbol *i* represents each level.

Table 3.10 Variance analysis of $L_9(3^4)$ orthogonal experiment on C/N ratio of culture medium.

Source of variance	Mycelial dry weight (g/L)			*F*-ratio	HA content (mg/L)			*F*-ratio
	SS	df	MS		SS	df	MS	
Sucrose	0.76	2	0.38	0.56	19.38	2	9.69	0.10
Maltose	2.60	2	1.30	1.92	41.12	2	20.56	0.21
Peptone	7.46	2	3.73	5.51	78.07	2	39.03	0.40
NH_4NO_3	39.08	2	19.54	28.89*	2217.89	2	1,108.94	11.46*
Errors	10.82	16	0.676		1548.85	27	96.80	

SS: Sum of squares; df: Degree of freedom; MS: Mean of square.

* *F*-ratio $> F_{0.05}$

It was found that the combination $A_1B_1C_3D_1$ obtained slightly lower HA content than that of the control BPD broth (Table 3.11). It indicates that complex carbon and nitrogen sources in the testing conditions have no contribution to obtain high yield of HA. Excessive carbon and nitrogen sources probably might inhibit production of HA. Considering result of single factor selection for carbon source and nitrogen source in 3.4.4 and 3.4.5, therefore, 3% maltose and 1% NH_4NO_3 were selected as the best carbon and nitrogen sources in broth of HA production.

Table 3.11 Mycelial weight and HA content obtained from repeated experiment for C/N ratio in *S. bambusicola* GZAAS2.0629 fermentation.

Treatment	Mean mycelial weight (g/L)	Mean HA content (mg/L)
BPD broth	6.18	58.45
$A_1B_1C_3D_1$*	3.43	57.37

* $A_1B_1C_3D_1$ = sucrose 20%, maltose 20%, peptone 30%, and NH4NO3 10%.

3.5.7 Inorganic Salt Selection for HA Production

Inorganic elements are components of microorganism cells and involve the metabolism promoting synthesis of secondary metabolites. In this study, factors and levels design of the orthogonal layout and the experimental result were shown in Table 3.12. As shown in Table 3.13, $CuSO_4 \cdot 5H_2O$, KH_2PO_4, and $MgSO_4 \cdot 7H_2O$ showed a slight positive effect on mycelial dry weight especially the KH_2PO_4, but other factors with the negative effects. Moreover, $CuSO_4 \cdot 5H_2O$ and KH_2PO_4 showed a positive effect on HA yield and $CuSO_4 \cdot 5H_2O$ had a higher contribution on HA yield than that of KH_2PO_4. The best combination of inorganic salts on mycelial dry weight

should be $A_2B_2C_2D_1E_1F_1$, but that on HA production was $A_2B_2C_1D_1E_1F_1$, namely 0.001% $CuSO_4 \cdot 5H_2O$ and 0.2% KH_2PO_4. While $FeSO_4 \cdot 7H_2O$ also had larger R value, but it was a negative effect indicating that Fe^{2+} would probably inhibit the mycelial growth and HA production. Magnesium (Mg^{2+}) is generally reported important for microorganism, which serves as a cofactor for many enzymes, complexes with ATP, and stabilizes ribosomes and cell membranes (Prescott et al., 1999). As one of the basic elements of many fermentation media, $MgSO_4$ has not been found to contribute to HA or other PQD production in many reports (Zhang et al., 2002; Cai et al., 2004; Shi et al., 2004; Chen et al., 2005a; Liang et al., 2008; Li et al. 2002). Furthermore, $MgSO_4 \cdot 7H_2O$ in this study showed a little negative effect on HA yield. $CaCl_2$ and $NaNO_3$ also showed a negative effect on the mycelial growth and HA production. No statistical differences among the 6 testing inorganic salts were observed (Table 3.14). Therefore, 0.001% $CuSO_4 \cdot 5H_2O$ and 0.2% KH_2PO_4 were selected as the best inorganic salt for HA-yielding medium.

Table 3.12 Result of L_8 (2^6) orthogonal test of mycelia biomass and HA production of *S. bambusicola* GZAAS2.0629 cultured in varied combination of inorganic salts at 26°C with 120 rpm for 13 days.

Run	A*	B	C	D	E	F	Mycelial dry weight (g/L)	HA content (mg/L)
1	1	1	1	1	1	1	7.47 ± 0.61	46.62 ± 1.14
2	1	1	1	2	2	2	6.73 ± 0.84	28.02 ± 6.55
3	1	2	2	1	1	2	7.78 ± 0.26	36.01 ± 1.27
4	1	2	2	2	2	1	7.43 ± 0.37	34.09 ± 5.27
5	2	1	2	1	2	1	7.86 ± 0.16	48.08 ± 2.42
6	2	1	2	2	1	2	6.93 ± 0.10	38.02 ± 2.84
7	2	2	1	1	2	2	7.28 ± 0.51	51.95 ± 5.47
8	2	2	1	2	1	1	7.84 ± 1.83	46.80 ± 0.70

* A, B, C, D, E and F represent factors of $CuSO_4 \cdot 5H_2O$, KH_2PO_4, $MgSO_4 \cdot 7H_2O$, $FeSO_4 \cdot 7H_2O$, $CaCl_2$, and $NaNO_3$ of 2 levels as indicated in Table 3.4 respectively. Data given are means of three replications ± SD.

Table 3.13 Analysis of inorganic salt on mycelial biomass and HA production of *S. bambusicola* GZAAS2.0629 with L_8 (2^6) orthogonal test.

	Mycelial dry weight (g/L)						HA content (mg/L)					
	A	B	C	D	E	F	A	B	C	D	E	F
k 1	7.40	7.29	7.37	7.67	7.57	7.72	36.19	40.19	43.35	45.67	41.86	43.90
k 2	7.48	7.59	7.50	7.21	7.30	7.16	46.21	42.21	39.05	36.73	40.54	38.50
R	0.08	0.30	0.13	0.46	0.27	0.56	10.02	2.02	4.30	8.94	1.32	5.40

k_i^X = Mean of the factor at each level. $R_i^X = \max k_i^X - \min k_i^X$.

X represents factor A, B, C, D, E or F respectively, and symbol i represents each level.

Table 3.14   Variance analysis of L_8 (2^6) orthogonal experiment on inorganic salt of culture medium.

Source of variance	Mycelial dry weight (g/L)			F-ratio	HA content (mg/L)			F-ratio
	SS	df	MS		SS	df	MS	
CuSO$_4$·5H$_2$O	0.09	1	0.09	0.14	603.61	1	603.61	38.77
KH$_2$PO$_4$	0.67	1	0.67	1.03	24.64	1	24.64	1.58
MgSO$_4$·7H$_2$O	0.18	1	0.18	0.27	110.94	1	110.94	7.13
FeSO$_4$·7H$_2$O	0.80	1	0.80	1.23	479.18	1	479.18	30.78
CaCl$_2$	0.20	1	0.20	0.30	10.51	1	10.51	0.68
NaNO$_3$	1.34	1	1.34	2.05	174.74	1	174.74	11.22
Errors	9.11	14	0.65		217.96	14	15.57	

SS: Sum of squares; df: Degree of freedom; MS: Mean of square.

3.5.8 Selection of Bamboo Addition in the Medium for HA Production

Bamboo addition was a main factor of HA production. Without bamboo addition, the fungal inocula turned into brown mycelia balls, which had tight structure and difficult to grow in the broth. The mycelial weight increased with the increasing amount of bamboo added. As shown in Figure 3.7, HA content also increased with the addition amount of the bamboo powder until it arrived 10 g/L in the broth. The result of statistical analysis (Table 3.15) showed that the treatment 10 g/L of bamboo addition was significantly different ($p < 0.05$) from that of the treatment with 5 g/L, but there was no difference among that of the 10 g/L, 15 g/L, and 20 g/L treatments. Therefore, 10 g/L would be the best amount of bamboo addition in the broth.
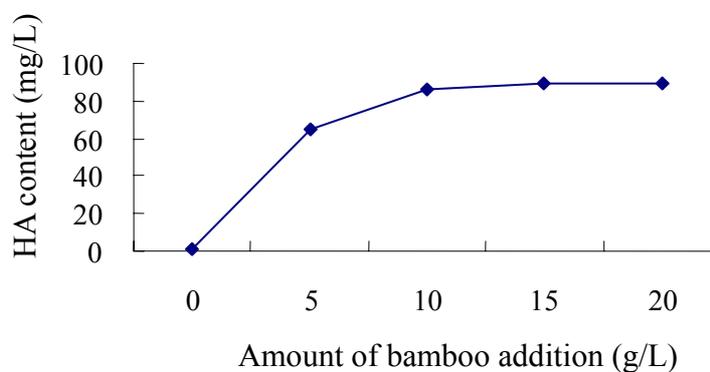
Figure 3.7 Effect of bamboo addition on HA production of *S. bambusicola* GZAAS2.0629 cultured at 26°C with 120 rpm for 13 days.

Based on our knowledge, bamboo as a component of medium for HA production was reported only once by Zhang et al. (2002), but they did not mention the relationship between the bamboo addition and HA yield. In this study, the bamboo addition in the fermentation broth was proved playing an important role on HA production.

Table 3.15 Effect of bamboo addition on mycelial growth and HA production of *S. bambusicola* GZAAS2.0629 cultured at 26°C with 120 rpm for 13 days

Bamboo powder (g/L)	Mycelial dry weight* (g/L)	HA content* (mg/L)
20	5.77 ± 0.07a	89.30 ± 2.79a
15	5.70 ± 0.41b	89.23 ± 14.16a
10	5.39 ± 0.58c	85.75 ± 15.56a
5	4.37± 0.09c	64.38 ± 0.78b
0	0.70± 0.06c	1.40 ± 0.22c

* Mean of three replications ± SD, followed by a common letter in each column are not significantly different by SNK at 5% level.

3.5.9 Initial pH Selection for HA Production

With raise of initial pH value of the fermentation broth, the mycelial dry weight increased continuously, but the absorbance at 465 nm and the HA content did not follow the trend. As shown in Figure 3.8, HA yield was affected by different initial pH of the broth. With raise of pH value from 5.0 to 7.0, the HA content increased gradually and arrived the highest at pH 7.0, and subsequently decreased. However, there was no significant difference among those pH treatments.

Comparing to other reports on HA production, the initial pH values of media were quite different. Cai et al. (2004) and Shi et al. (2004) considered pH 5.5 to 6 was the best initial pH. Chen, et al. (2005a) suggested pH 6.0 was the best one. And Liang et al. (2008) indicated the best initial pH value was 7.5. In this study, pH 7.0 should be a good treatment for both mycelia weight and absorbance at 465 nm. Whereas considering reduction work of adjustment on pH value, the unadjusted pH about 5.5 was selected as the initial pH.

Figure 3.8   Effect of initial pH of fermentation broth on HA production of *S. bambusicola* GZAAS2.0629 cultured in optimized medium at 26°C with 120 rpm for 13 days.

3.5.10   Selection of Inoculum Amount for HA Production

Inoculum amount is not only a factor influencing growth of mycelium, but also affects on emergence and accumulation of secondary metabolites. Smaller inoculum amount would prolong fermentation cycle due to shortage of starting inoculum. When the inoculum amount is too high, growth of the mycelium would be restrained (Shi, 1989). In this study, inoculum amount of 2, 4, 6, 8, and 10 pieces of mycelial mat were tested but no significant difference on HA yield was observed (Figure 3.9). The highest HA content was obtained in the treatment with 4 pieces of inocula. The mycelial weight obtained was also the highest in this treatment. The absorbance at 465 nm was similar among each treatment. The different HA contents were mainly caused by the difference of the mycelial weight. Therefore 4 pieces of the inoculum were selected as suitable amount for HA production.

Figure 3.9   Effect of inoculum amount on HA production of *S. bambusicola* GZAAS2.0629 cultured in optimized medium at 26°C with 120 rpm for 13 days.

3.5.11 Selection of Fermentation Temperature for HA Production

In the 4 temperature treatments, the fungus nearly stopped growth at 31°C. The highest HA content was obtained in the treatment of 25°C, followed by that of 27°C and 29°C. As shown in Figure 3.10, with the temperature increasing from 25 to 27°C, the absorbance at 465 nm had a clear decline, but the mycelial weight continuously increased. The result indicates that the suitable temperature for increasing of mycelial biomass was not advantageous to obtain high HA content. The temperature at the point of intersection in Figure 3.10 should be better than the others. Therefore, 26°C was selected as fermentation temperature.
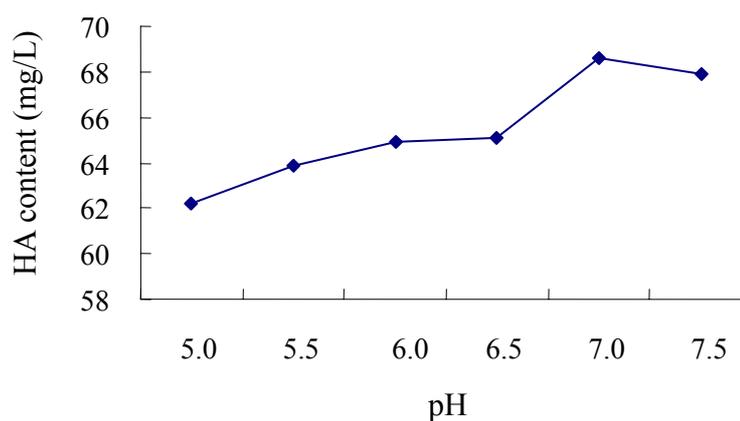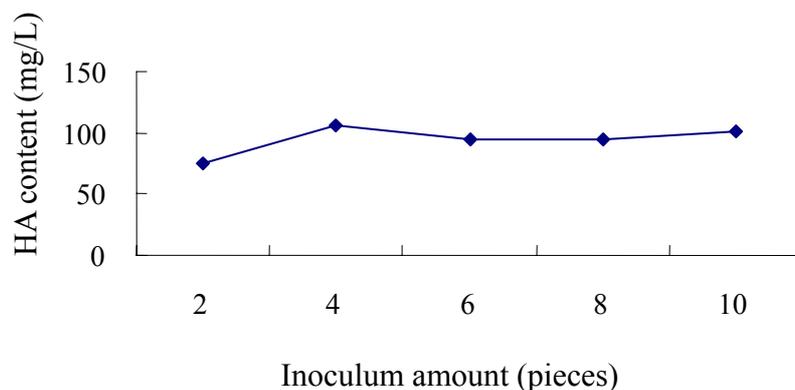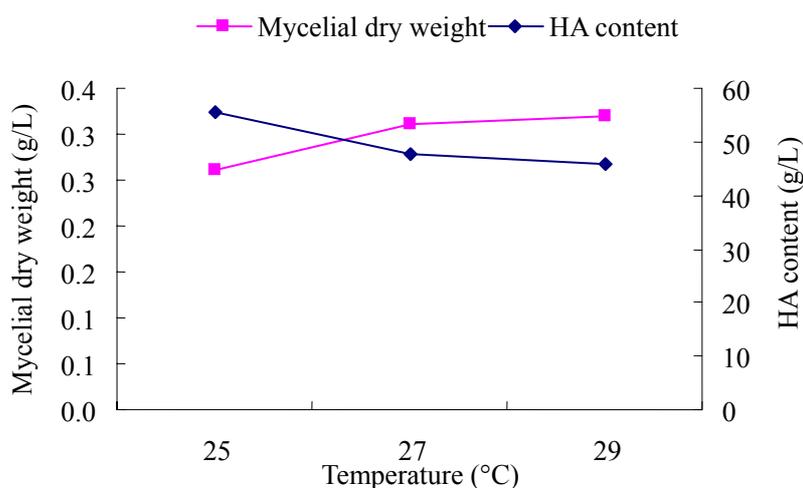

Figure 3.10 Effect of temperature on HA production of *S. bambusicola* GZAAS2.0629 cultured in optimized medium at 26°C with 120 rpm for 13 days.

3.5.12 Hypocrellin Production under the Improved Conditions

Based on previous experiments selecting on components of the medium and fermentation conditions, the optimized conditions for high HA yield

were 3% maltose, 1% NH_4NO_3, 0.001% $CuSO_4 \cdot 5H_2O$, 0.2% KH_2PO_4, 20% potato, 1% bamboo powder, unadjusted pH, with inoculum amount of 4 pieces of mycelial mat per 50 ml broth, shaking at 120 rpm and incubation at 26°C for 13 days. Under this condition, an experiment was conducted. The HA content obtained from the repeated experiment was 112 mg/L, which is more than 2 times of that with basal broth.

3.5.13   Overall Discussion

According to this study, the fermentation isolate GZAAS2.0629 with the highest HA production was screened out from 32 isolates. Its HA yield was 112 mg/L under the optimized conditions. Comparing to other reports, the results were different from each other including hypocrellin yield, fermentation time, incubation conditions, and nutrition requirement for high yield of hypocrellin. Under the liquid state fermentation condition, about 50 mg/L of HA in experiment of single factor screening was obtained by Shi et al. (2004), but that from Zhang et al. (2002) was 7.6 mg/L. Chen et al. (2005b) found that 8 g/L hypocrellin ($E_{1cm}^{1\%}$ 465 = 52) was produced in the optimum medium components and incubating conditions. Under the solid state fermentation conditions, about 2.4 chromo-value of the total perylenequinones from 1 g mycelia was obtained (Li et al., 2003a), but 2.02 mg HA production from 1 g dry solid substrate was reported by Liang et al. (2008). Among these reports, the determination methods and quantity units were different making it difficult for the comparison. Furthermore, although those fermentation strains were described as *S. bambusicola* or *Shiraia* sp. by the authors, their morphological characteristics were rather different from that of our isolates. It is waiting to be proved in the future whether they are the same species.

3.6 Conclusion

Thirty two isolates of *S. bambusicola* were divided into two groups based on their growth rate. The isolates GZAAS2.0703 and GZAAS2.0707 were selected as representatives in sampling time experiment and found that the 11th to 13th day were suitable sampling times to screen for high HA-producing isolates. The isolate GZAAS2.0629 was found to produce the highest HA at the three sampling days among all the testing isolates, which was screened out as the fermentation isolate. The improved fermentation condition for HA production was identified as using the broth containing 3% maltose, 1% NH_4NO_3, 0.001% $CuSO_4 \cdot 5H_2O$, 0.2% KH_2PO_4, 20% potato, 1% bamboo powder, unadjusted pH, with inoculum amount of 4 pieces of mycelial mat (4mm diameter) per 50 ml, shaking at 120 rpm, and incubation at 26°C for 13 days. Under these fermentation condition, the HA content obtained was 112 mg/L.

CHAPTER IV

CONCLUSION AND RECOMMENDATION

In this study, biology of *S. bambusicola* and screening for high HA-producing isolates and optimum fermentation conditions were carried out and obtained results as following:

1. Stroma of *S. bambusicola* grew only on top stalks of the previous year branches without any evidence of parasitic relationship with the bamboo. It grew around the interspace of bamboo leaf sheaths with tissue specificity on leaf sheath of several bamboo genera.

2. The fungus *S. bambusicola* was confirmed to have bitunicate asci with pseudoparaphyses thus should be placed in the Pleosporales order and not in the Dothideales.

3. Asexual and sexual developments of *S. bambusicola* were observed and for the first time, 4 stages of asci and ascospores development were described. The 4 stages consisted of ascus primordium formation, ascus elongation, young ascospore formation, and ascospore maturation.

4. No sporulation could be found in the study conditions.

5. The conidia and ascospores produced in the present year were the infection source of next year.

6. A developed capillary method was found to be the best for obtaining single-spore isolates.

7. The fungus was successfully inoculated and cultivated on *Phyllostachys rubromarginata* bamboo after being sprayed with fresh conidium suspension. The fungus had a considerably long incubation period of at least 10 months.

8. Thirty two isoltaes were obtained from the isolation including 10 single spore isolates, 11 stromatal tissue isolates and 11 multispore isolates. From those isolates, GZAAS2.0703 and GZAAS2.0707 were selected as representatives to find that the 11th to 13th day were suitable sampling time to screen for high HA-producing isolates.

9. The isolate GZAAS2.0629 was found to produce the highest HA at the three sampling days among all the testing isolates, which was screened out as the fermentation isolate.

10. The optimized conditions for high HA yield were 3% maltose, 1% NH_4NO_3, 0.001% $CuSO_4 \cdot 5H_2O$, 0.2% KH_2PO_4, 20% potato, 1% bamboo powder, unadjusted pH, with inoculum amount of 4 pieces of mycelial mat per 50 ml broth shaking at 120 rpm and incubation at 26°C for 13 days. Under this condition, the HA content was 112 mg/L

11. The content of HA even though was still high when the optimized condition was repeated but it was not as high as that of the initial experiment. This could have resulted from the deterioration of the fungal inoculum. Therefore, more research is needed to investigate the preservation methods of this fungus for future use.

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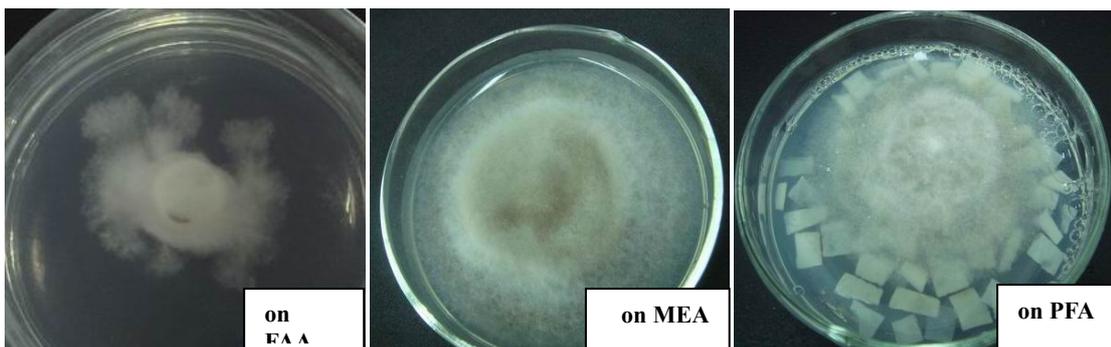
APPENDIX

Appendix figure 1 Stromata of *S. bambusicola* (the yellow parts on stroma are conidial masses).
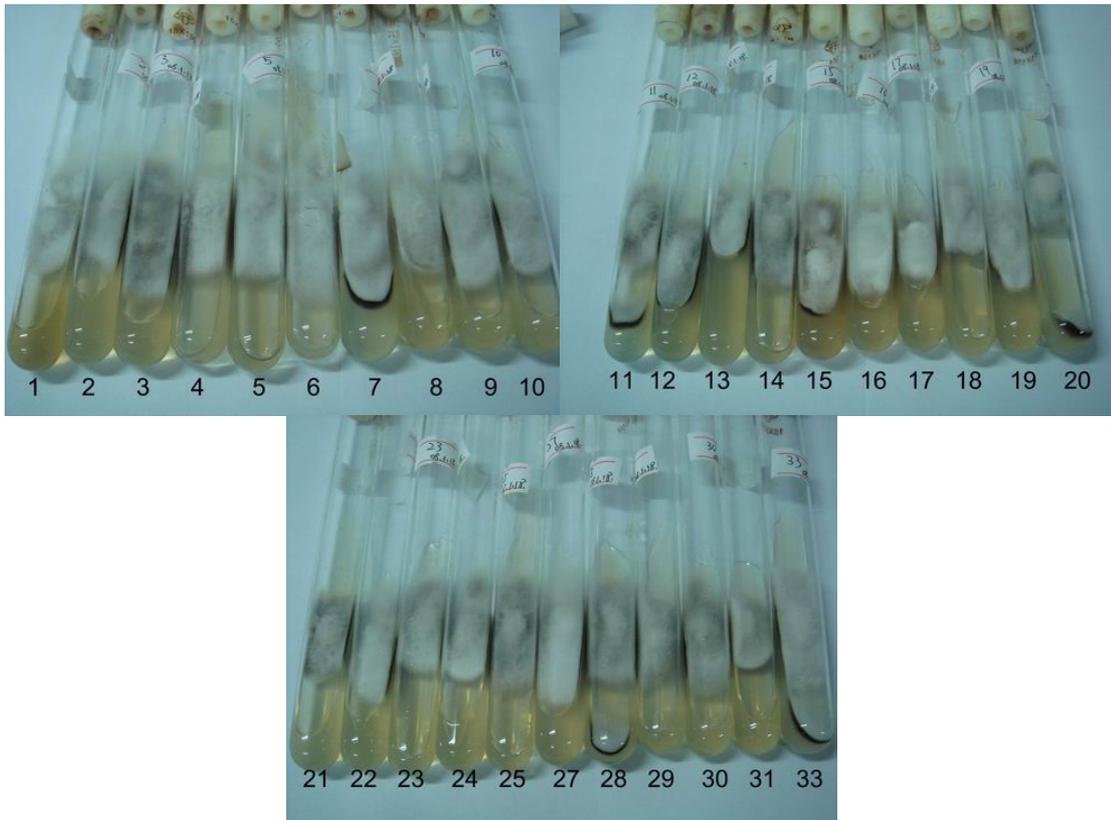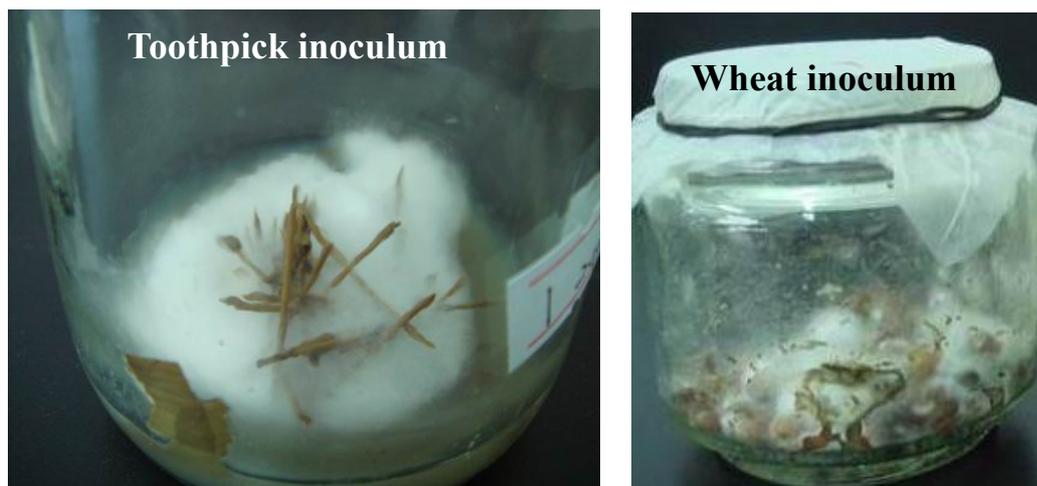
Appendix figure 2   *S. bambusicola* isolates on PDA slants.

Appendix figure 3   Colony of *S. bambusicola* on different media.

Appendix figure 3   Primordia of *S. bambusicola* on PDA slant and dry BPDA plate.

Appendix figure 4   Toothpick inocula and wheat inocula used in artificial inoculation.

BIOGRAPHY

Yongxiang Liu was born on February 11, 1978 in Guizhou province, the People's Republic of China. She earned Bachelor's Degree in agriculture from Deparptment of Plant Protection, Agricultural Institute of Guizhou University in 2001 and was conferred Master's Degree in microbiology from Guizhou University in 2005. Then she worked at Guizhou Academy of Agricultural Sciences. In 2006, she started to study for Ph.D. under the supervision of Dr. Sopone Wongkaew at School of Crop Production Technology, Suranaree University of Technology, Thailand.