ชีววิทยาและการปลูกเชื้อรา *Ustilaginoidea viren* (Cooke) Takahashi ในข้าว

นายเหอ ไหหยง

วิทยานิพนธ์นี้สำหรับการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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BIOLOGY AND ARTIFICIAL INOCULATION OF *Ustilaginoidea virens* (Cooke) Takahashi IN RICE

He Haiyong

A Thesis Submitted in Fulfillment of the Requirements for the

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BIOLOGY AND ARTIFICIAL INOCULATION OF *Ustilaginoidea virens* (Cooke) Takahashi IN RICE

Suranaree University of Technology has approved this thesis submitted in fulfillment of the requirements for a Master's Degree.

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เหอ ไหหยง : ชีววิทยาและการปลูกเชื้อรา *Ustilaginoidea viren* (Cooke) Takahashi ในข้าว (BIOLOGY AND ARTIFICIAL INOCULATION OF *Ustilaginoidea virens* (Cooke) Takahashi IN RICE) อาจารย์ที่ปรึกษา : ดร.โสภณ วงศ์แก้ว, 54 หน้า.

วัตถุประสงค์ของงานวิจัยครั้งนี้คือ 1) เพื่อศึกษาชีววิทยาของเชื้อ *Ustilaginoidea virens* 2) เพื่อพัฒนาอาหารที่เหมาะกับการผลิตหัวเชื้อ *U. virens* 3) เพื่อพัฒนาวิธีการปลูกเชื้อสำหรับการศึกษาความต้านทานของข้าวต่อโรคดอกกระถินโดยเก็บตัวอย่างข้าวที่เป็นโรคจำนวน 70 ตัวอย่างจาก 7 อำเภอในเขตมณฑลกุ๋ยโจว ประเทศสาธารณรัฐประชาชนจีน ในระหว่างปี ค.ศ. 2007-2008 จากนั้นนำมาแยกเชื้อสปอร์เดี่ยวจำนวน 138 ไอโซเลต นำมาทดสอบวิธีการเก็บรักษาจำนวน 6 วิธีพบว่า การเก็บโดยวิธีย้ายเชื้อเป็นระยะ (periodic transfer) และวิธีปิดทับเชื้อด้วยพาราฟิน (paraffin oil overlay) เป็นวิธีเก็บรักษาที่ดีที่สุดสามารถเก็บเชื้อได้นานกว่า 15 เดือน อันเป็นระยะเวลาที่สิ้นสุดการทดลอง ขณะที่การเก็บในเมล็ดข้าวเปลือก เชื้อสูญเสียความมีชีวิตภายในระยะเวลาเพียง 2 เดือน การทดสอบเลี้ยงเชื้อ *U. virens* ไอโซเลตที่สุ่มมาจากประชากรรวมบนอาหารเหลว 7 ชนิด พบว่าอาหาร potato sucrose broth (PSB) สามารถผลิตโคนิเดียได้เข้มข้นมากที่สุดคือ $7.25x10^7$ โคนิเดีย.มล$^{-1}$ ภายในเวลา 9 วัน ขณะที่อาหาร corn broth (CB) ผลิตได้ต่ำสุดคือ $1.47x10^4$ โคนิเดีย.มล$^{-1}$ ภายในเวลาที่เท่ากัน การทดลองปลูกเชื้อให้กับข้าวพันธุ์ Gangxiang 707 โดยใช้เชื้อ *U. virens* 2008-33-1 เปรียบเทียบ 2 วิธี ระยะการเจริญของข้าว 3 ระยะ พบว่าการฉีดสารแขวนลอยของโคนิเดียให้กับข้าวในช่วงปลายของระยะตั้งท้อง (late booting stage) ทำให้เกิดโรคสูงสุดคือ 50.43% ขณะที่การใช้วิธีฉีดพ่นโคนิเดีย (conidia spraying) ที่ระยะเดียวกัน ทำให้เกิดโรคเพียง 34.75% การทดสอบความสามารถในการก่อโรคของเชื้อ *U. virens* จำนวน 8 ไอโซเลต กับข้าวพันธุ์ Zhongyou 177 โดยใช้วิธีฉีดด้วยสารแขวนลอยของโคนิเดียให้กับข้าวช่วงปลายของระยะตั้งท้อง พบว่าเชื้อมีระดับความรุนแรงในการก่อโรคแตกต่างกันโดยที่ไอโซเลต 2008-11-1 ให้คะแนนความรุนแรงสูงสุดระดับ 9 และทำให้ต้นข้าวที่ทดสอบเป็นโรคจำนวนสูงสุดถึง 81.66% ขณะที่ไอโซเลต 2007-48-1 ให้คะแนนความรุนแรงระดับ 5 และจำนวนต้นข้าวที่เป็นโรคจำนวนต่ำสุดเพียง 15.57% เมื่อนำเชื้อ *U. virens* ทั้ง 8 ไอโซเลตนี้ไปทดสอบกับข้าวจำนวน 6 พันธุ์ โดยใช้วิธีปลูกเชื้อวิธีเดียวกัน พบว่าข้าวทุกพันธุ์อ่อนแอหรืออ่อนแอปานกลางต่อเชื้อ *U. virens* ยกเว้นพันธุ์ Fengyouxiangzhan ที่อ่อนแอมากต่อเชื้อ *U. virens* ไอโซเลต 2007-79-1 และพันธุ์ Nongfengyou 256 ที่ต้านทานปานกลางต่อเชื้อไอโซเลตเดียวกัน เมื่อทำการประเมินปฏิกิริยาของพันธุ์ข้าวต่อเชื้อ *U. virens* ทุกไอโซเลตพบว่า Fengyouxiangzhan Jixiangyon 830 และ Gangyou 827 ให้จำนวนต้นข้าวที่เป็นโรคเพียง 23.85% การประเมินระดับความรุนแรงของเชื้อทั้ง 8 ไอโซเลตพบว่า ไอโซเลต 2008-33-1 มีระดับความรุนแรงสูงสุดโดยทำให้ข้าวเป็นโรค 58.09% ขณะที่ไอโซเลต 2008-2-2 มี

ระดับความรุนแรงต่ำสุด ทำให้ข้าวเป็นโรคเพียง 28.19% พันธุ์ข้าวที่มีการแสดงออกดีที่สุด คือ Nongfengyou 256 ที่ต้านทานปานกลางต่อเชื้อ จำนวน 12.5% และอ่อนแอปานกลางต่อเชื้อ จำนวน 87.5% จากเชื้อทั้งหมดที่ทดสอบ

สาขาวิชาเทคโนโลยีการผลิตพืช
ปีการศึกษา 2555

ลายมือชื่อนักศึกษา_____
ลายมือชื่ออาจารย์ที่ปรึกษา_____
ลายมือชื่ออาจารย์ที่ปรึกษาร่วม_____

HE HAIYONG : BIOLOGY AND ARTIFICIAL INOCULATION OF *Ustilaginoidea virens* (Cooke) Takahashi IN RICE. THESIS ADVISOR : SOPONE WONGKAEW, Ph.D., 54 PP.

BIOLOGY/ARTIFICIAL INOCULATION/*Ustilaginoidea virens*/RICE

Objectives of the research were 1) to study the biology of *Ustilaginoidea virens*, 2) to develop suitable media for *U. virens* inoculum production, and 3) to develop an artificial inoculation technique for the study of false smut resistance in rice. Seventy diseased rice samples were collected from 7 districts of Guizhou province, China during 2007-2008 and 138 single-spore isolates of *U. virens* were isolated. Among the 6 preservation methods tested, periodic transfer of viable culture and paraffin oil overlay were the two best methods that kept the culture viable for as long as 15 months which is when the experiment ended, while preserving in rice grain, the fungus lost its viability within 2 months. Among 7 media tried on one randomly selected *U. virens* isolate, potato sucrose broth (PSB) produced the highest concentration of conidia at 7.25×10^7 conidia. ml^{-1} in 9 days while corn broth (CB) gave the lowest concentration of only 1.47×10^4 conidia. ml^{-1} in the same period. Among 2 inoculation methods applied at 3 different rice growth stages of Gangxiang 707 rice variety using spore suspension of 2008-33-1 *U. virens* isolate, conidia injection at late booting stage produced the highest false smut incidence of 50.43% while conidia spraying during the same period gave only 34.75% disease incidence. Pathogenicity test of selected 8 *U. virens* isolates on Zhongyou 177 rice variety using the conidia injection at late booting stage, showed that the isolates were different in their aggressiveness in which isolate 2008-11-1 gave the highest virulence score of 9

with the disease incidence of 81.66% while isolate 2007-48-1 gave the lowest score of 5 with only 15.57% disease incidence. These 8 *U. virens* isolates were further tested on 6 rice varieties using the same inoculation technique. Most of the rice varieties were either susceptible or moderately susceptible except Fengyouxiangzhan that was highly susceptible to *U. virens* isolate 2007-79-1 and Nongfengyou 256 was moderately resistant. When the evaluation was done across all the *U. virens* isolates, Gangyou 827, Jixiangyou 830 and Fengyouxiangzhan had the highest disease incidence of 55.06%, 54.79% and 53.50%, respectively while Nongfengyou 256 had only 23.85%. For the pathogen, 2008-33-1 was the most aggressive giving 58.09% disease incidence while 2008-2-2 gave the lowest of 28.17%. Nongfengyou 256 performed best, being moderately resistant to 12.5% of the *U. virens* isolates and moderately susceptible to 87.5% of them.

School of Crop Production Technology

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Student's Signature_____

Advisor's Signature_____

Co-advisor's Signature_____

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He Haiyong

TABLE OF CONTENTS

Page

TABLE OF CONTENTS (Continued)

Page

TABLE OF CONTENTS (Continued)

Page

LIST OF TABLE

LIST OF TABLE (Continued)

LIST OF FIGURES

Figure **Page**

LIST OF ABBREVIATIONS

ANOVA	=	Analysis of variance
CB	=	Corn broth
CEI	=	Comprehensive evaluation index
CEIS	=	Comprehensive evaluation index scores
CRD	=	Complete randomized design
CV	=	Coefficient of variation
DI	=	Disease incidence
DIS	=	Disease incidence score
DMRT	=	Duncan's multiple range test
EC	=	Emulsifiable concentration
HR	=	Highly resistant
HS	=	Highly susceptible
MDI	=	Mean disease incidence
MR	=	Moderately resistant
OB	=	Oatmeal broth
PDA	=	Potato dextrose agar
PDB	=	Potoato dextrose broth
PSB	=	Potato sucrose broth
R	=	Resistant
RGB	=	Rice grain broth

LIST OF ABBREVIATIONS (Continued)

RILs	=	Recombinant inbred lines
S	=	Susceptible
SC	=	Suspension concentration
SDS	=	Smut ball density scoring
SPSS	=	Statistical Package for the Social Sciences
U. virens	=	*Ustilaginoidea virens*
YP-PDB	=	Yeast extract peptone potato dextrose broth

CHAPTER I

INTRODUCTION

1.1 General introduction

The fungus *Ustilaginoidea virens* (Cooke) Takahashi (teleomorph *Villosiclava virens*) causes false smut of rice (*Oryza sativa* L.) and corn (*Zea mays* L.) in humid areas (Mulder and Holliday, 1971; Lee and Gunnell, 1992; Abbas, Sciumbato and Keeling, 2002) and was discovered by Cooke in 1878.

The rice false smut is a worldwide disease and has been regarded as a minor rice disease throughout major rice-growing countries in the world before 1970's (Deng, 1989; Yaegashi, Fujita and Sonoda, 1989; Sugha *et al*., 1992). It has been found in many countries, such as China, India, Japan, Italy, Australia, Philippines, Brazil and Mayanma (Ou, 1972; Dodan, 1996). With the change of weather condition, large application of nitrogen fertilizer and large-scale planting of hybrid rice, the rice false smut has become more and more serious. It has already changed from a minor disease to a major disease in Guizhou and all rice growing areas in China, and many rice-growing countries in Asia since 1970. In 1982, the disease had spread more than 666,000 hectares in Hunan, China. The epidemic area had increased from 200,000 to 330,000 hectares from 1984-1996 at Liaoning, China (Ji, 2002). In 1993, the disease was reported to increase from 60,000 to 100,000 hectares in Yunnan, China (Liao and Li, 1994), equal to 13.7% of the total rice production. The disease incidence was 10-30%, but in some serious fields it could be as high as 50-60%. Up to 39 false smut

balls could be found on each rice plant. The rice yield was reduced 5-30% as a result of infection in Guizhou. The false smut balls have toxin including ergot alkaloid toxin that can cause rumination stopping in cows, suppress the tubulin of mammals and cause necroses of liver, kidney, and bladder tissues in mice (Dhindsa, Ulakh and Chahal, 1991; Nakamura and Izumiyama, 1992; Chib, Tikoo and Kalha, 1992; Iwasaki, 1992; Yukiko *et al*., 1994; Nakamura, Izumiyama and Ohtsubo, 1994; Li *et al*., 1995; Ji, 2000; Sinha and Singh, 2003). Therefore, the rice false smut not only threatens rice production in yield and quality but also produces toxins that are dangerous to the health of human and livestocks.

Both domestic and international researchers have done a lot of work and some achievements on the fungus which includes its biological characteristics, major outbreak period, inoculum sources, and infection cycle have been reached. Lu *et al.* (1996) reported artificial culture conditions of temperature, carbon source, and pH value. However, there have been few reports on optimal sporulation culture conditions and component of culture media, single spore isolation and conservation methods. There have been some studies on disease resistance using artificial inoculations (Zhang *et al*., 2003; Liu, Chen and Zhang, 2007), but there were different results of disease incidence and lower reproducibility. At present there have not been an established artificial inoculation technique and evaluation criteria for disease resistance to rice false smut. Therefore, make it difficult to study the resistance of rice to this disease.

Selecting and using resistant varieties are the most cost-effective measures to control plant diseases. In recent years, some investigations have been done in Guizhou. Results showed that the disease incidence of rice false smut were

significantly different among rice varieties. For example, in 2008, the disease incidence and disease index of various rice varieties were 1.07% to 39.19% and 0.32 to 16.77, respectively. The differences observed among the varieties were 36.6 times and 52.4 times. In order to effectively control rice false smut by using resistant varieties, the study of biological characteristics leading to the successful artificial inoculation is most important.

1.2 Objectives of the research

1.2.1 To study the biology of *U. virens.*

1.2.2 To develop suitable medium for inoculum production of *U. virens.*

1.2.3 To develop an artificial inoculation technique suitable for the study of false smut resistance in rice.

1.3 Scope of the research

The experiments were conducted under laboratory and greenhouse conditions using *U. virens* isolates collected in Guizhou. Rice varieties used in the experiment were those that are available in Guizhou province.

1.4 Anticipated outcomes

1.4.1 Obtain a proper method for single spore isolation, preparing inoculum and preserving the cultures.

1.4.2 Obtain the most suitable artificial inoculation technique

1.4.3 Obtain a proper method for evaluating rice varieties resistant to false smut.

CHAPTER II

LITERATURE REVIEWS

2.1 Classification of rice false smut

The rice false smut fungus can be classified as follows :

Kingdom: Fungi

Phylum: Ascomycota

Class: Ascomycetes

Subclass: Sordariomycetes

Order: Hypocreales

Family: Clavicipitaceae

Genus: *Villosiclava*

Species: *virens*

Anamorph : *Ustilaginoidea virens*

(http://en.wikipedia.org/wiki/Ustilaginoidea_virens)

True smut is caused by fungi in Basidiomycetes, but the sexual stage of rice false smut belongs to *Villosiclava virens* Tanaka (Tanaka *et al*., 2008), in Ascomycetes that is why it is called false smut. The asexual stage of rice false smut belongs to *Ustilaginoidea virens*, in Deuteromycetes.

2.2 Symptoms and morphological characteristics of *Ustilaginoidea virens*

2.2.1 Symptoms on rice panicle

False smut damages the rice plant by infecting the ovaries of the rice kernel in its early development (Webster and Gunnell, 1992). Once inside, the fungus takes over the ovary and replaces it with spores that burst, producing a large orange ball between the glumes. These balls are covered with spores that spread the disease. Rice false smut (green smut) is a common grain disease in most rice-growing areas of the world. Infection of the fungus transforms individual grains of the panicle into greenish spore balls (false smut balls) with a velvety appearance, the spore balls are almost smooth when young, and become warty and dark-green while the spore balls are mature. The ball surface is covered by powdery dark-green chlamydospore, conidia and mycelia. A sclerotium can sometime appear on the surface of smut balls (Ou, 1972).

If rice varieties have low disease resistance to false smut, more rainfall and large application of nitrogen fertilizer, the rice panicles will be infected by *U. viren* and produce spore balls fully packed with mycelia and chlamydospores. The symptom of rice false smut is rather special, because except for rice panicle, the plants do not express any other symptoms. The spore balls are 8-12 times bigger than the rice grains and have a velvety appearance. The surfaces of spore balls were covered with a lot of chlamydospores. Initially, the color of the spore balls is orange, then become brown or yellow green or green black when the rice are matured (Fig. 1) (Lee and Gunnell, 1992; Kim and Park, 2007).

2.2.2 Morphological characteristics

The asci of *Villosiclava virens* are hidden inside the stroma. The ascospores are thread shaped (Fig. 2). The asci can germinate and produce ascospores. Perithecia are produced annulosly and in monolayer embeded in the rind of stroma. The apex of perithecia grow out of stroma surface and papillae structure were formed. When asci are fully developed, the apical wall of perithecia disappear, and asci emerged from the perithecia. The ascospore is hyaline, unicellular, and filiform. The chlamydospore is spherical or elliptical, yellowish-brown to black-brown, with thick and compacted wal1. The surface of chlamydospore has many verrucae. Both ascospore and chlamydospore form secondary conidia when they germinate. The secondary conidia have similar morphological characteristics as the submerged conidia obtained from liquid culture. They are thin-wall conidia, hyaline, with smooth surface, directly produce next generation of the thin-wall conidia when they germinate. This type of reproduction process can be repeated successively (Zhang *et al*., 2003).

2.3 Life cycle of *Ustilaginoidea virens*

The life cycle is as indicated in Fig 3. The false smut fungus can survive as sclerotia and hardened spore balls, known as pseudomorph which can survive up to 4 months under field conditions (Kim and Park, 2007). In the following year, the sclerotia can germinate to produce conidia, infect the panicle and transform them individualy into greenblack spore balls. The surfaces of false smut balls are covered with abundant powdery dark-green chlamydospores (Ou, 1972). They can germinate to produce a lot of secondary conidia and hypha. The secondary conidia are round to elliptical and warty on the surface with diameters approximately ranging from 3-5μm,

and are globose to irregularly rounded.

Preliminary studies suggest that roots can be infected by pathogen of *U. virens* (Schroud and TeBeest 2005). Shen (2004) reported the major infection period was 7 days before flowering. The rice panicles are infected by conidia and produce smut balls containing abundant chlamydospores. The chlamydospores germinate to produce large amount of hypha and conidia or turn to sclerotia. After the sclerotia and chlamydospores overwinter, they germinate to produce more conidia and infect rice panicles.

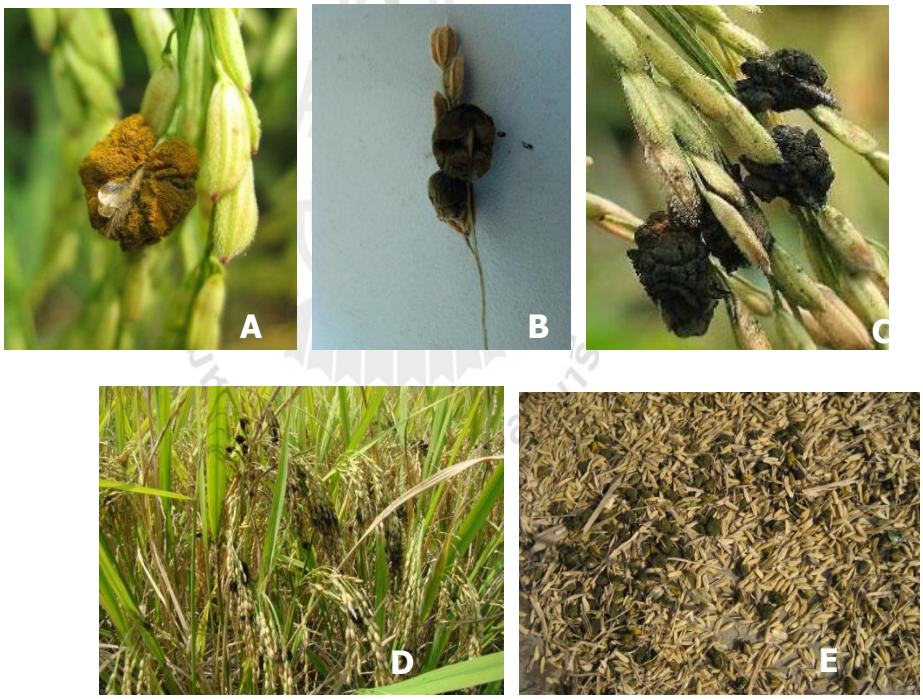

Figure 1. Symptoms of false smut on rice panicles (A) orange stage, (B) brown stage, (C) green black stage, (D) field symptoms, (E) smut balls on harvested grains.

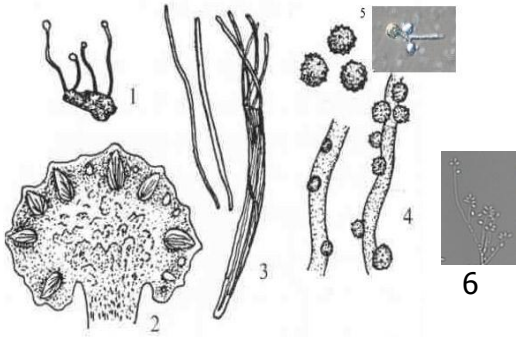

Figure 2. Morphological characteristic of *Ustilaginoidea virens* (1) sclerotium and its germination, (2) stroma and asci, (3) ascospores, (4) hypha and chlamydospore, (5) germinating chlamydospores, (6) hypha and conidia. http://image.baidu.com)

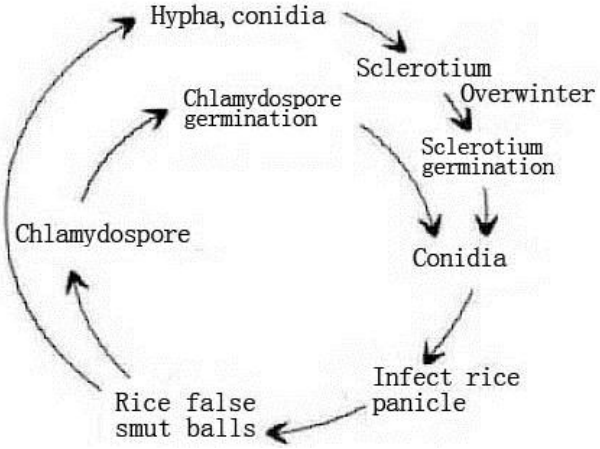

Figure 3. Life cycle of *Ustilaginoidea virens* (http://image.baidu.com)

2.4 Economic importance of rice false smut

In recent years, the rice false smut has become a serious problem in part due to the change in climate and rice varieties. The disease occurs in more than 40 countries, such as China, India, Japan, Italy, Australia, Philippines, Brazil and Mayanma (Ou,

1972; Dodan and Singh, 1996). Especially in the many rice-growing countries in Asia (Li *et al.*, 2008), but also in the U. S (Brooks *et al*., 2009). It has already changed from a minor disease to a major disease. In 1988, rice production was severely damaged by infection of *U. virens* in the northern areas of Japan (Yaegashi *et al*., 1989). This disease has become one of the most important fungal diseases in rice planting regions of Guizhou and all rice growing areas in China and other parts of the world, because japonica varieties of rice are widely cultivated, most of which are very susceptible (Deng, 1989). In 1982, the disease had been reported in Hunan, Liaoning, Yunnan, China (Ji, 2002; Liao and Li, 1994). The disease incidence was 10-30%, but in some serious fields it could be as high as 50-60%. Up to 39 false smut balls could be found on each rice plant. The rice yield was reduced 5-30% as a result of infection in Guizhou, China (http://nongyao.aweb.com.cn). Infected rice panicle forming a ball of mycelia and always destroyed by the disease, the outermost layers are spore-producing (Webster and Gunnell, 1992). The rice false smut not only causes decreasing yield and quality, but also produce ustiloxin in the false smut balls. It is an inhibitor of microtubules (Luduena *et al.*, 1994) that can affect the health of human and livestock (Koiso *et al.*, 1994). It can cause rumination stopping of cows, suppress the tubulin of mammals and cause necroses of liver, kidney, and bladder tissues in mice (Nakamura and Izumiyama, 1992; Dhindsa, ulakh and Chahal, 1991; Chib, Tikoo and Kalha,1992; Sinha, Sinha and Singh, 2003).

2.5 Control of rice false smut

At present, cultural, physical, biological and chemical control have been used to manage rice false smut disease. For examples, the diseased straws and sclerotia

were removed from rice fields after harvest. Using suitable plant spacing, application appropriate rate of nitrogen fertilizer, utilizing clean rice seeds have also been applied. Ditmore and TeBeest (2006) reported that false smut is often considered seed-borne. For the biological control. Paenibacillus wettable powder (WP) had been applied to control rice false smut (Liu, *et al*., 2007). Li *et al.* (2008) reported Paenibacillus Xi-55 against *U. virens*, the research results showed that the average inhibition rate against spore germination of *U. virens* was 69.33%. Ma *et al*. (2008) reported that the bamboo tar was an agricultural fungicide. Their experimental results showed the efficiency of bamboo tar against *Ustilaginoidea virens* was 56.3%~73.6%. For the chemical control, application of Difenoconazole emulsifiable concentration (EC), Hexaconazole suspension concentration (SC) and Validamycin·suspension concentration (SC) were reported. Chen *et al.* (2009) reported that the control effect of 30% Difenoconazole or Propiconazole EC against rice false smut was up to 67.60% and 81.71%. Xiong *et al.* (2009) reported the controlling efficacy of 30% Hexaconazole SC against rice false smut was up to 77.09% in the field. Wen *et al*. (2010) reported the controlling efficacy of 10% Validamycin suspension concentration (SC) could be up to 80%. These research results showed that the biological control methods were not highly effective. Although chemical control can be highly effective, but it could have bad effects on the environment. More research have been emphasized on using resistant varieties to control rice false smut (Chen, 1992 and Ji, 2000). Therefore, screening and growing resistance varieties will be the best method to control false smut.

2.6 Research on rice plant resistance to *Ustilaginoidea virens*

At present, to effectively controlling the rice false smut, studies on disease

resistance by artificial inoculation have been conducted by many researchers. The domestic and international researchers have done a lot of work about isolation and preservation, biological characteristics, major epidemic period, inoculum sources and infection cycle. Liu *et al.* (2009) reported the successful rate of sclerotium germination was up to 92.3%, but they could obtain pure isolates only at 8.5% and 5.3% through tissue transplanting and chlamydospores suspension methods respectively. They also found that the optimum pH value in the culture media should be at 6. Lu, Dai and Zhou (2009) reported that potato dextrose agar (PDA) and the rice extract medium were the solid media most suitable for the fungal growth. The potato dextrose broth (PDB) was the best liquid medium for sporulation. The condition of light had no effect on the fungal sporulation. Ahonsi *et al.* (2000) carried out disease resistance experiment to false smut on rice, his results showed rice varieties had different reaction to false smut. ITA150, ITA315, Agbede and ITA335 were the most susceptible varieties to false sumut, the mean disease incidence (MDI) was 36.1, 40.5, 40.6 and 43.3%, respectively. Sonoda (1992) reported varietal difference in resistance of rice to false smut too. Jiang *et al.* (2010) research results showed that among the 35 varieties tested, there were no varieties with high resistance. Lu *et al.* (1996) reported the artificial culture conditions of temperature, carbon source, pH value, and the environmental conditions suitable for infection, these results showed that optimum temperature and pH values range was 25~28ºC and 5.8 to 6.98 respectively, the humidity more than 90% and sugar can promote the pathogen to infect rice plants. At the same time, some researchers showed that high relative humidity (>90%) (Biswas, 2001) and temperature ranging from 25 to 35ºC favored the disease (Dodan and Singh, 1996). Liao and Wang (1994) reported the

spray inoculation with conidiospore suspension of *U. virens* at 7-14 days before rice heading stage, and soil inoculation with chlamydospor powder which was collected from diseased kernels in field in the previous year before transplanting. The results suggested these techniques could be used for the identification of the resistance to *U. virens* of rice cultivars. Ashizawa (2011) reported using conidia suspension of *U. virens* to inject into the leaf sheath of rice plant, the rice panicle can be infected. The results suggested the inoculation method can be useful for determining the resistance level of rice varieties to false smut. To study the inheritance of the resistance to rice false smut, Li *et al.* (2008) using a population of 157 recombinant inbred lines (RILs) of F10 derived from the cross between resistant cultivar IR28 (*Oryza sativa* subsp. Indica) and susceptible landrace Daguandao (*O. sativa* subsp. japonica) was analyzed using the mixed major genes and polygenes inheritance model. The results showed that the resistance to rice false smut was controlled by the mixed 2 major genes and polygenes model (model E-1–3). The 2 major genes had equivalent additive effect of 11.41, and their heritability was about 76.67%. The heritability of polygenes was about 22.86%. Both effects of major genes and polygenes should be considered in the resistance breeding to rice false smut. Zhang *et al.* (2003) and Liu *et al.* (2007) did some studies on disease resistance to rice false smut by artificial inoculation. Their results showed that chlamydospores collected from previous year and kept in -20℃ could not cause the disease. Du *et al.* (1992) and Dai *et al.* (2011) reported that conidia were the major source of infection not chlamydospores. Wang *et al.* (1996) reported the disease incidence of injection was higher than that of the spray inoculation with the conidia of *U. virens*. They also found that the booting stage was most susceptible to *U. virens* and potato extract could increase the percentage of

disease panicles when it was added into the inoculum. They also found that rice plants in weather condition of prolonged rains and less sunshine had higher disease severity index.

CHAPTER Ⅲ

MATERIALS AND METHODS

3.1 Outline of the experiments

The experiments were carried out as outlined in Figure 4

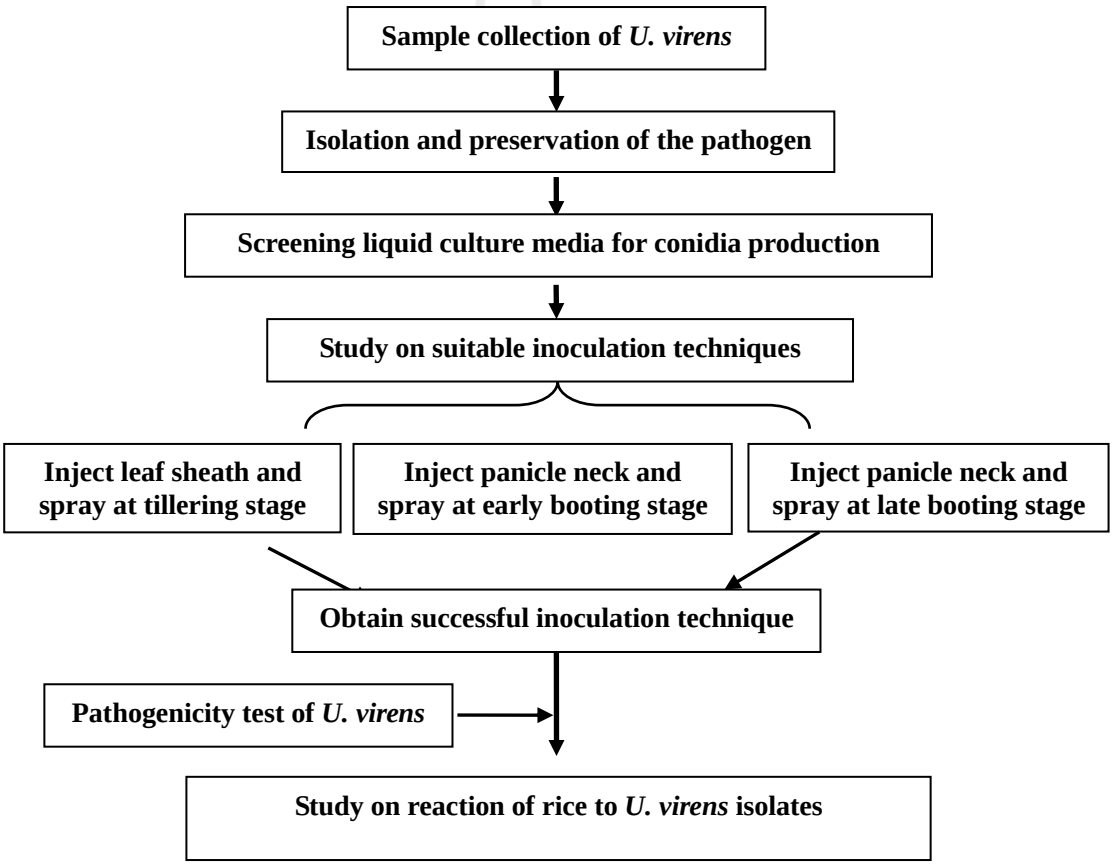

Figure 4. Outline of the experiments

3.2 Sample collection and isolation of the fungal pathogen

Samples of the rice false smut balls were collected from 7 districts in Guizhou in 2007-2008 which were Zunyi, Guiyang, Tongren, Qiandongnan, Qiannan, Anshun and Xingyi.

To isolate the causal agent, the diseased samples were washed thoroughly under distilled water and dried under a lamina flow in the laboratory. Subsequently, the smut balls from each location were separately surface sterilized with 75% ethanol (EtOH) for 25 seconds. The excess traces of EtOH on the balls were removed by washing 2 times in sterile distilled water and then transferred aseptically into a flask containing sterile distilled water and 10 glass beads. The cultured flasks were then incubated and shaked at 28±2°C in the dark for 2 min after that 2 ml of the culture suspension was spread on surface of potato sucrose agar (PSA: potato 200 g, sucrose 20 g, agar 17 g, distilled water 1000 ml) (Liu *et al*., 2009). The PSA plates were incubated at 28±2°C for few hours and were examined frequently under a microscope for germinating single spores which were then marked with ink on the plate surface. These single spores were aseptically transferred with a sterile cork borer to fresh PSA plates and incubated at 28±2°C for 10 days. The pure single spores were then transferred into PSA slants and maintained for the future experiments. To maintain the culture, the fungus that was isolated from respective geographical region and maintained as a separate isolate, was sub-cultured on PSA slants and allowed to grow at 28±2°C for two weeks. Subsequently the slants were preserved in a refrigerator and renewed once every two months (Fig. 5).

3.3 Preservation methods

Six preservation methods were tried in this experiment as follows (Fig. 6):

3.3.1 Periodic transfer of active culture

The fungal isolates were kept in PSA slants in a refrigerator at 4°C and periodically transferred to fresh slants at 3 month interval or longer period. The viability and sporulation were checked periodically.

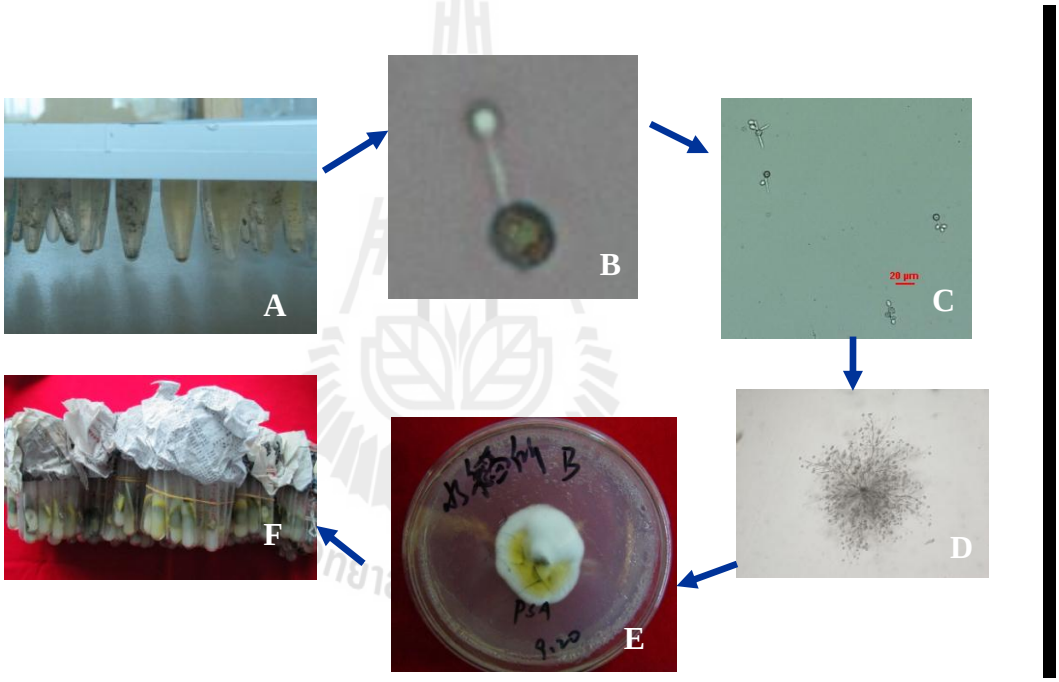

Figure 5. Single spore isolation of *U. virens*. (A) chlamydospore suspension, (B) germinating chlamydospores, (C) germinatiing chlamydospre and conidia, (D) colony and conidia, (E) colony, (F) slant culture.

3.3.2 Paraffin oil overlay preservation

The isolates were transferred to PSA slants and incubated at 28±2°C for 15 days in the dark. After the mycelia fully covered the medium surface, pour sterile liquid paraffin to completely cover the mycelia. In a parallel condition the isolates were transferred into PSB in a vial (potato sucrose broth) and incubated at 28±2°C for 9 days. After the mycelia fully grown, the sterile liquid paraffin was poured into the culture and preserved in refrigerator at 4°C. The cultures preserved in both forms were checked at 3 month interval for the viability and sporulation.

3.3.3 Preservation in rice grain

Rice grains were soaked in water for 24 h to absorb enough water, boiled for 30 min, put into test tubes, and autoclaved for 20 min. After excess water drainage, the isolates were transferred into the tubes and incubated under 28±2°C for 20-30 days in the dark until the rice grains dried completely and preserved within desiccator with silica gels. The culture were checked at 3 month interval for the viability and sporulation.

3.3.4 Preservation in glycerol

The fungal pathogens of each isolate were transferred into PSB in a vial with the same condition as 3.3.2. After the mycelia fully grown, pour sterile 10% or 100% glycerol to completely cover the mycelia. Subsequently the slant were preserved in a refrigerator at 4°C and was checked at 3 month interval for the viability and sporulation.

3.4 Screening liquid culture media for conidia production

To obtain optimal liquid culture condition for sporulation, 7 media of different composition were tested by shaking culture at 150 rpm. The cultures were incubated under 28±2°C for 9 days. Subsequently, the samples were taken out at third day interval to measure the spore concentration using hematocytometer until the spore number had reached its peak in each medium. One isolate of *U. virens* was randomly picked as the representative isolate for the investigations. The experiment was carried out in a complete randomized design (CRD) in 4 replications. The best liquid medium would be selected to be used for conidia production in the inoculation experiment. The 7 liquid media (Li, *et al*. 2008) were as follows:

Media A : Potato dextrose broth [PDB] (potato 200 g, dextrose 20 g, water 1000 ml).

Media B : Potato sucrose broth [PSB] (potato 200 g, sucrose 20 g, water 1000 ml).

Media C : Yeast peptone potato dextrose broth [YP-PDB](potato 200 g, dextrose 20 g, yeast extract 0.1 g, peptone 0.1 g, water 1000 ml).

Media D : Oatmeal broth [OB] (oatmeal 30 g, water 1000 ml).

Media E : Corn broth [CB](corn 200 g, water 1000 ml).

Media F : Rice broth [RB] (unpolished rice 200 g, water 1000 ml).

Media G : Rice grain broth [RGB] (rice grain 30 g, water 1000 ml)

3.5 Study on suitable inoculation techniques

3.5.1 Inoculum prepareation

The randomly selected fungal isolates were used in this experiment. Details of

the isolates are as shown in Table 1. Spore suspensions of the respective fungal isolates were prepared having approximately 1×10^6 conidia/ml (Fig. 7). For the injecting inoculation, tween 80 was added into the spore suspension prior to use but for the spraying inoculation, 0.5% gelatin was added prior to use to prevent spore dessication.

Table 1. *Ustilaginoidea virens* single spore isolates used in the experiment

Isolate	**Origin (District)**	**Isolate**	**Origin (District)**
2008-1-3	Zunyi	2007-6-1	Qiandongnan
2007-79-1	Zunyi	2007-66-1	Qiandongnan
2008-2-2	Guiyang	2008-11-1	Qiannan
2007-11-1	Guiyang	2008-33-1	Anshun
2007-48-1	Tongren	2008-36-1	Xinyi

Figure 6. Preservation of *U. virens* for the experiments. (A) periodic transfer, (B) paraffin oil overlay on mycelia and conidia, (C) paraffin oil overlay slant culture, (D) rice grain, (E) mycelia and conidia preserved in 10% glycerol, (F) mycelia and conidia preserved in 100% glycerol

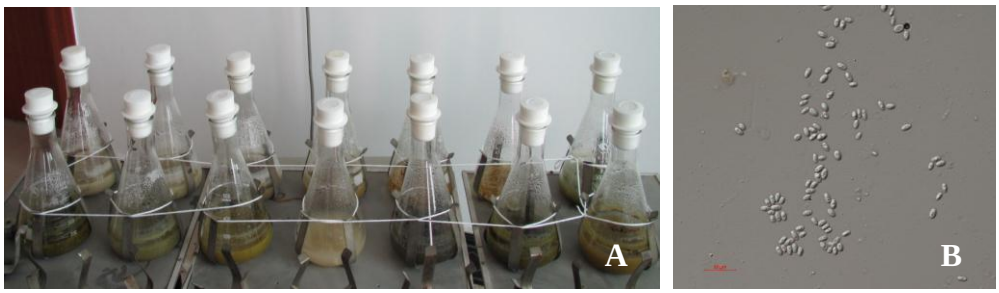

Figure 7. Inoculum preparation. (A) shake culture, (B) conidia suspension

3.5.2 Rice plant preparation

Rice seeds of variety Gangxiang 707 were soaked in warm water at 60°C for 1 h to kill seed-borne pathogens and left overnight to absorb water. After that, the seeds were pre-germinated at 30°C for 36 h before planting in a small field plot. At twenty-five days after planting, seedlings were transplanted into cement plots, 90×80 cm in size, in the greenhouse, 25 seedings per plot (Fig. 8).

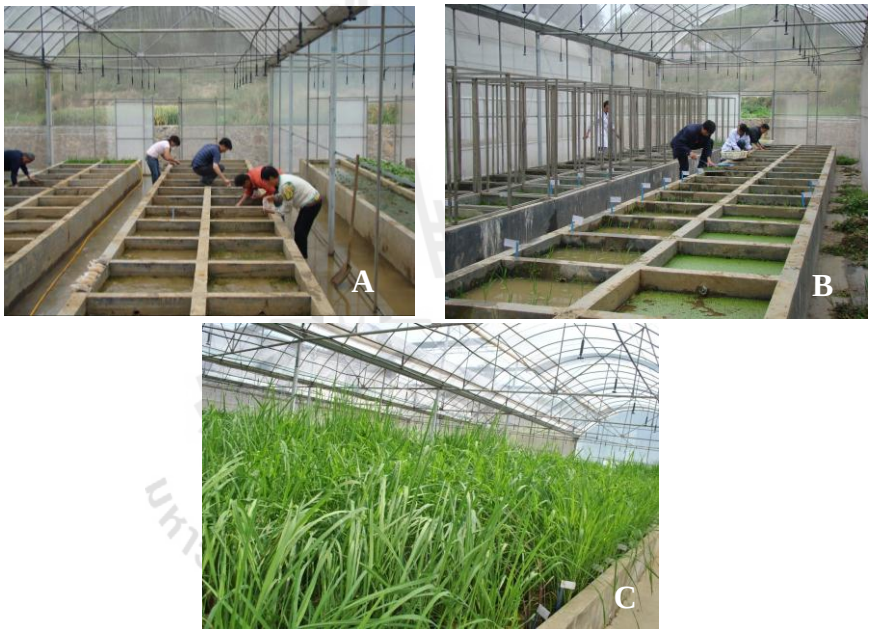

Figure 8. Rice plant preparation. (A and B) seeding, (C) rice plants at the time of inoculation

3.5.3 Artificial inoculation of rice

The spore suspension of isolate 2008-33-1 (1×10^6 spore/ml) was used for inoculating the healthy Gangxiang 707 rice plants. Two different artificial inoculation methods, conidia suspension spraying and conidia injection were investigated at

tillering, booting and late booting stages. For conidia spraying, the suspension was sprayed on leaf surface until run–off by an air compressor. For conidia injection, 1 ml of the suspension was injected into the upper part of leaf sheath covering the young panicle of each test plant using a syringe. In another set, instead of spore suspension only sterile distilled water was sprayed or injected to serve as a negative control. The experiment was conducted as factorial in CRD with 4 replications. Subsequently the inoculated rice plants were kept in moist condition and observations were made at regular intervals for symptom development within 3 days after the inoculation.

3.6 Pathogenicity test of the *Ustilaginoidea virens* isolates

To confirm and prove pathogenicity of the 8 *U. virens* isolates, spore suspension of each fungal isolate at 1×10^6 spore/ml were inoculated into healthy Zhongyou 177 rice cultivar at the late booting stage using the conidia injection technique. In another set, instead of spore suspension only sterile distilled water was injected to serve as a negative control. The experiment was conducted as factorial in CRD with 4 replications. Observations were made at regular intevals for symptom development within 3 days. The virulence level of each fungal isolate was evaluated based on the percentage of disease incidence classified into different scores as indicated in Table 2.

3.7 Reaction of rice varieties to *Ustilaginoidea virens* isolates

Eight rice varieties of different resistant levels and from different growing areas as shown in Table 3 were used. The plants were prepared as described in 3.5.2. The inoculation was done by conidia injection technique at late booting stage (5~7

days before flowering). Selected eight virulent fungal isolates from section 3.6 were prepared in sterile distilled water as spore suspensions. The experiment was conducted as factorial in CRD with 4 replications. Observations were made at regular intervals for symptom development within 3 days. Number of infected rice plants and grains were counted at 40~50 days after the inoculation (maturity stage). The disease incidence, disease index and density of rice smut balls were calculated and evaluated as follows :

$$\text{Disease incidence (\%)} = \frac{\text{Total infected panicles}}{\text{Total inoculated plants}} \times 100$$

The rice false smut incidence and density of smut ball were divided into 6 scoring scales based on Zhang *et al.* (2006) (Table 2).

The disease index were calculated according to the amount of rice smut balls of each score and corresponding value of scale (Table 2) as follows.

$$\text{Disease index} = \frac{\sum(\text{infected panicles of each rating} \times \text{rating value})}{\text{Total panicles} \times \text{The highest rating value}} \times 100$$

The comprehensive evaluation index (CEI) was calculated as follows :

$$\text{CEI} = [(\text{score of disease incidence} \times 60) + (\text{score of smut ball density} \times 40)]/100$$

(Ministry of Agriculture of P. R. China, 2006)

Subsequently，the CEI was used for the resistance evaluation of the rice varieties using the criteria in Table 4.

3.8 Statistical analysis

Treatment effects on most experiments were analysed using ANOVA by the SPSS program. In some experiments, the data were arcsine transformed before the analysis. Duncan's multiple range test (DMRT) at $p \leq 0.05$ was used to separate treatment means.

Table 2. Scoring criteria for categorizing virulence level of *U. virens* isolates

Scoring	**Disease incidence(%)**	**Smut ball density (No/panicle)**
0	≤1%	0
1	＞1 but≤5%	1
3	＞5 but≤10%	＞1 but≤5
5	＞10 but≤25%	＞5 but≤10
7	＞25 but≤50%	＞11 but≤15
9	＞50%	＞16

Table 3. Rice varieties and their observed reaction to *U. virens* employed in the study

Rice variety	**Area of origin**	**Reaction to false smut**
Gangxiang 707	Sichuan	S
Zhongyou 177	Sichuan	R
Gangyou 827	Sichuan	S
Heyou 6	Sichuan	R
Suaiyoulianhe 2	Guizhou	R
Jinxiangyou 830	Guizhou	S
Fengyouxiangzhan	Jiangshu	S
Nongfengyou 256	Anhui	R

R=resistant S=susceptible

Table 4. Scoring of the comprehensive evaluation index (CEI) of reaction to rice varieties of false smut disease

Scoring	**CEI**	**Resistant level**
0	0	Highly resistant (HR)
1	≤1	Resistant (R)
3	＞1 but≤3	Moderately resistant (MR)
5	＞3 but≤5	Moderately susceptible (MS)
7	＞5 but≤7	Susceptible (S)
9	＞7	Highly susceptible (HS)

CHAPTER IV

RESULTS

4.1 Samples collection and single spore isolation of the pathogen

To obtain *U. virens* in the test, 70 rice diseased panicle samples were collected in Guizhou province during 2007~2008. From the samples, 138 single spore isolates had been isolated from different regions (Table 5). There were 10 samples and 16 single spore isolates from Zunyi, 13 and 26 from Guiyang, 6 and 14 from Tongren, 11 and 24 from Qiandongnan, 10 and 21 from Qiannan, 8 and 23 from Anshun, and 8 and 14 from Xinyi respectively.

Table 5. Single spore isolates of *U. virens* collected from various districts of Guizhou Province, China

District	**Date of collection (year)**	**No. of sample**	**No. of single spore isolate**
Zunyi	2007-2008	10	16
Guiyang	2007-2008	13	26
Tongren	2008	6	13
Qiandongnan (Southeast)	2008	11	24
Qian nan (South)	2008	10	21
Anshun	2007-2008	12	23
Xinyi	2008	8	15
Total	—	70	138

4.2 Preservation of *Ustilaginoidea virens*

Among six methods studied, the results showed that the periodic transfer was the best one, the period of survival was more than 15 months. The second best method was paraffin oil overlay in which the fungus could survive for 12-15 months. Preservation in glycerol ranked the third in which two fungus isolates could survive for 9~11 months. The rice grain gave the lowest survival period of less than 2 months (Table 6). Therefore, the periodic transfer and the paraffin oil overlay were the best preservation methods for *U. virens*.

Table 6. Survival period of *U. virens* preserved by different methods

Preservation method	**Pathogen form**	**Survival period (month)**
Periodic transfer	Colony on slant culture	>15
Paraffin oil overlay	Hypha and conidia	12-15
Paraffin oil overlay	Colony on slant culture	13-15
Rice grain	Colony on grain	<2
100% Glycerol	Hypha and conidia	10-11
10% Glycerol	Hypha and conidia	9-10

4.3 Screening liquid culture media for conidia production

After samples from 7 liquid culture media were taken out to measure the spore concentration until the spore number had reached its peak. The results showed that no conidia appeared when the cultures were taken out after 3 days, but at 5 days conidia started to be seen in the PSB and PDB media. The spore concentration were 2.3×10^3 and 3.5×10^2 conidia/ml respectively. The conidia concentration went up to 4.28×10^5

and 7.25×10^7 conidia/ml in the PSB liquid medium after 7 and 9 days respectively, indicated that PSB was the best medium for conidia production (Table 7). The second best was PDB liquid medium. The spore concentration in this medium went up to 3.45×10^4 and 5.38×10^6 conidia/ml, when the culture period reached 7 and 9 days respectively. Among the 7 media, the CB appeared to be less suitable for conidia production.

Table 7. Conidia concentration of *U. viren* cultured in different liquid medium at different incubation time

Liquid medium	**Concentration(conidia. ml^{-1})[1] /Time (day)**			
	3	**5**	**7**	**9**
PDB	0	3.48×10^2b	3.35×10^4b	5.36×10^6b
PSB	0	2.29×10^3a	4.23×10^5a	7.20×10^7a
YP-PDB	0	0c	2.86×10^2f	1.23×10^5d
OB	0	0c	4.63×10^3c	1.87×10^5c
CB	0	0c	2.92×10^2f	1.47×10^4f
RB	0	0c	4.50×10^2e	3.71×10^4e
RGB	0	0c	6.42×10^2d	4.02×10^4e
F-test	-	**	**	**
CV (%)	-	24.79	39.6	32.4

[1] The data were Log transformed before analysis. Means in the the same column followed by different letters are significantly different at P≤0.05 by DMRT.

Note: PDB: potato dextrose broth, **PSB:** potato sucrose broth, **YP-PDB:** yeast extract peptone PDB, **OB:** oatmeal broth, **CB**: corn broth, **RB:** unpolished rice broth, **RGB:** rice grain broth.

4.4 Study on suitable inoculation technique

In the experiment, 40 rice panicles were used per replication. The average disease incidences of spraying inoculation were 0.00, 21.18, and 34.75% at tillering, early booting and late booting stages respectively. The disease incidence was much higher when rice plants were inoculated by conidia injection (Fig. 9) which had 0.00, 38.50 and 50.43% at tillering, early booting and late booting stages respectively (Table 8). Result from combined analysis showed clearly that conidia injection were the best inoculation technique (Table 9) and it should be done at the late booting stage (Table 10).

Table 8. False smut incidence on Gangxiang 707 rice variety inoculated by 2 different methods at 3 growth stages

Inoculation method	**Inoculation stage**	**Disease incidence (%)** [1]
Spraying inoculation	Tillering stage	0.00 e
	Early booting stage	21.18 d
	Late booting stage	34.75 c
	Blank control	0.00 e
Injecting inoculation	Tillering stage	0.00 e
	Early booting stage	38.50 b
	Late booting stage	50.43 a
	Blank control	0.00 e
F-test		**
CV(%)		12.86

[1] The data were arcsine transformed before the analysis. Means in the same column followed by different letters are significantly different at P≤0.05 by DMRT.

Table 9. Combined analysis of false smut incidence on Gangxiang 707 inoculated by 2 different methods at 3 growing stages

Inoculation method	**Disease incidence (%)** [1]
Spraying	13.98b
Injection	22.23a
F-test	**
CV(%)	8.12

[1] The data were arcsine transformed before the analysis. Means in the same column followed by different letters are significantly different at P≤0.05 by DMRT.

Table 10. Combined analysis of false smut incidence on Gangxiang 707 inoculated at 3 growth stages by different methods

Inoculation stage	**Disease incidence (%)** [1]
Tillering	0.00c
Early booting	29.84b
Late booting	42.59a
Blank control	0.00c
F-test	**
CV(%)	8.12

[1] The data were arcsine transformed before the analysis. Means in the same column followed by different letters are significantly different at P≤0.05 by DMRT.

4.5 Pathogenicity test of the *Ustilaginoidea virens* isolates

The results are shown in Table 11. Most of the *U. virens* isolates selected for the test could infect the Zhongyou 177 rice variety indicating that they all were pathogenic. Among them, isolate 2008-11-1 gave the highest disease incidence of 81.66% and its virulence can be put at score 9 which is the maximum according to the grading of disease incidence.

Table 11. The virulence of *U. virens* isolates to Zhongyou 177 rice cultivar

***U. virens* isolate**	**Disease incidence (%) [1]**	**Virulence score**
2008-1-3	33.05d	7
2008-2-2	33.72cd	7
2007-11-1	41.85c	7
2007-6-1	51.26b	9
2008-33-1	58.76b	9
2008-11-1	81.66a	9
2008-36-1	23.97e	5
2007-48-1	15.57f	5
CK	0.00g	
F-test	**	
CV(%)	8.98	

[1] The data were arcsine transformed before analysis. Means in the same column followed by different letter are significantly different at P≤0.05 by DMRT technique

4.6 Reaction of rice varieties to *Ustilaginoidea virens* isolates

The results were as shown in Table 12. Most of the rice varieties were susceptible or moderately susceptible to the *U. virens* isolates except variety Fengyouxiangzhan which was highly susceptible to the 2007-79-1 isolate while variety Nongfengyou 256 showed moderately resistant reaction to the same isolate. Combined analysis of all factors contributed to the disease reaction were presented in Tables 13, 14, and 15. Among the 6 rice varieties tested, Gangyou 827 appeared to have the highest disease incidence (55.06%) while Nongfengyou 256 seem to have the lowest (23.85%) (Table 13). Differences in virulence among the *U. virens* isolates were also observed in that isolate 2008-33-1 showed the highest disease incidence (58.09%) while isolate 2008-2-2 gave the lowest incidence (28.17%) (Table 14). When the overall disease reactions were analysed by combining all disease parameters, it appeared that Nongfengyou 256 performed the best by being moderately resistant to 12.5% of the *U. virens* isolates and only moderately susceptible to the left 87.5% (Table 15).

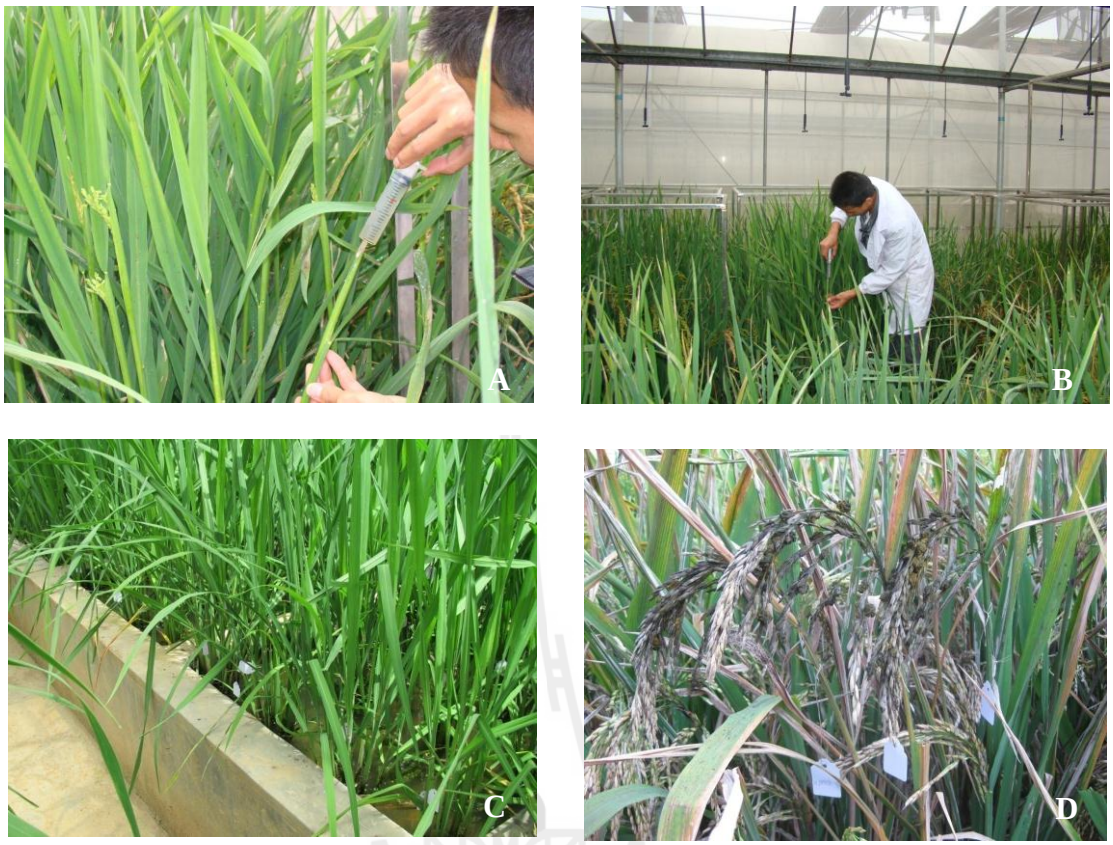

Figure 9. Artificial inoculation and symptom of rice false smut (A) and (B) conidia injection inoculation, (C) labelling of the inoculated plants, (D) symptoms of rice false smut developed from artificial inoculation

Table 12. Reaction of rice varieties to infection by *U. virens* isolates evaluated as smut ball density scoring (SDS), disease incidence (DI), disease incidence score (DIS), comprehensive evaluation index (CEI) and CEI scores (CEIS)

Rice variety	*U. virenes* isolates	SDS (ball/panicle)	DI(%) [1]	DIS	CEI	CEIS	Reaction
Gangyou	2008-2-2	1	34.72d	7	4.6	5	MS
	2008-33-1	3	80.78a	9	6.6	7	S
827	2007-11-1	3	55.44c	9	6.6	7	S
	2007-6-1	3	69.53ab	9	6.6	7	S
	2008-1-3	3	56.95c	9	6.6	7	S
	2008-36-1	1	42.05d	7	4.6	5	MS
	2007-66-1	3	60.27bc	9	6.6	7	S
	2007-79-1	1	40.77d	7	4.6	5	MS
F-test			**				
CV(%)			18.49				
Jinxiangyo	2008-2-2	3	39.11d	7	5.4	7	S
	2008-33-1	3	74.14a	9	6.6	7	S
u	2007-11-1	3	53.78c	9	6.6	7	S
	2007-6-1	3	74.14ab	9	6.6	7	S
830	2008-1-3	3	57.17c	9	6.6	7	S
	2008-36-1	1	40.67d	7	4.6	5	MS
	2007-66-1	3	60.11bc	9	6.6	7	S
	2007-79-1	1	39.23d	7	4.6	5	MS
F-test			**				
CV(%)			13.04				
Heyou 6	2008-2-2	1	14.94c	5	3.4	5	MS
	2008-33-1	3	42.12a	7	5.4	7	S
	2007-11-1	1	36.22ab	7	4.6	5	MS
	2007-6-1	1	42.05a	7	4.6	5	MS
	2008-1-3	1	32.90ab	7	4.6	5	MS
	2008-36-1	1	26.57b	5	3.4	5	MS
	2007-66-1	1	29.89ab	5	3.4	5	MS
	2007-79-1	1	31.55ab	7	4.6	5	MS
F-test			**				
CV(%)			30.05				

Table 12. Reaction of rice varieties to infection by *U. virens* isolates evaluated as smut ball density scoring (SDS), disease incidence (DI), disease incidence score (DIS), comprehensive evaluation index (CEI) and CEI scores (CEIS) (continued)

Rice variety	*U. virenes* isolates	SDS (ball/panicle)	DI(%) [1]	DIS	CEI	CEIS	Reaction
Suaiyou	2008-2-2	1	29.89ab	5	3.4	5	MS
	2008-33-1	3	37.66ab	7	5.4	7	S
lianhe 2	2007-11-1	1	37.73ab	7	4.6	5	MS
	2007-6-1	3	40.67a	7	5.4	7	S
	2008-1-3	1	31.39ab	7	4.6	5	MS
	2008-36-1	1	26.57bc	5	3.4	5	MS
	2007-66-1	1	17.89c	5	3.4	5	MS
	2007-79-1	1	17.89c	5	3.4	5	MS
F-test			**				
CV(%)			27.91				
Fengyou	2008-2-2	1	22.1d	5	3.4	5	MS
	2008-33-1	3	80.78a	9	6.6	7	S
xiangzhan	2007-11-1	3	49.33c	9	6.6	7	S
	2007-6-1	3	61.77b	9	6.6	7	S
	2008-1-3	3	49.33c	9	6.6	7	S
	2008-36-1	1	42.05c	7	4.6	5	MS
	2007-66-1	3	60.27b	9	6.6	7	S
	2007-79-1	5	62.31b	9	7.4	9	HS
F-test			**				
CV(%)			14.24				
Nongfengyou	2008-2-2	1	28.23ab	5	3.4	5	MS
	2008-33-1	1	33.06a	7	4.6	5	MS
256	2007-11-1	1	24.53ab	5	3.4	5	MS
	2007-6-1	1	21.59ab	5	3.4	5	MS
	2008-1-3	1	22.50ab	5	3.4	5	MS
	2008-36-1	1	24.53ab	5	3.4	5	MS
	2007-66-1	1	20.47b	5	3.4	5	MS
	2007-79-1	1	15.86b	3	2.2	3	MR
F-test			**				
CV(%)			40.92				

[1] The data were arcsine transformed before the analysis. Means in the same column followed by different small letters are significantly different at P≤0.05 by DMRT.

Table 13. Combined disease incidence of rice varieties inoculated with 8 *U. virens* isolates by conidia injection

Variety	**Disease incidence (%)** [1]
Gangyou 827	55.06a
Jinxiangyou 830	54.79a
Fengyouxiangzhan	53.50a
Heyou 6	32.01b
Suaiyoulianhe 2	29.96b
Nongfengyou 256	23.85c
F-test	**
CV(%)	16.74

[1] The data were arcsine transformed before the analysis. Means in the same column followed by different letters are significantly different at P≤0.05 by DMRT

Table 14. Combined disease incidence of 8 *U. virens* isolates inoculated to 6 rice varieties by conidia injection

Isolates	**Disease incidence (%)** [1]
2008-33-1	58.09a
2007-6-1	51.63b
2007-11-1	42.84c
2008-1-3	41.71c
2007-66-1	41.48c
2007-79-1	34.60d
2008-36-1	33.74d
2008-2-2	28.17e
F-test	**
CV(%)	16.74

[1] The data were arcsine transformed before the analysis. Means in the same column followed by different letters are significantly different at P≤0.05 by DMRT.

Table 15. Reaction of rice varieties to *U. virens* isolates

Variety	**Percentage of *U. virens* isolate with the reaction**					
	HR	R	MR	MS	S	HS
Gangyou 827	0	0	0	37.50	62.50	0.00
Jinxiangyou 830	0	0	0	25.00	75.00	0.00
Heyou 6	0	0	0	87.50	12.50	0.00
Suaiyoulianhe 2	0	0	0	75.00	25.00	0.00
Fengyouxiangzhan	0	0	0	25.00	62.50	12.50
Nongfengyou 256	0	0	12.5	87.50	0.00	0.00

Note: HR=highly resistant; R=resistant; MR=moderately resistant; S=susceptible; HS=highly susceptible.

CHAPTER V

CONCLUSION AND DISCUSSION

Results of the experiments can be concluded as follows.

5.1 Seventy diseased samples were collected from 7 districts of Guizhou province and 138 single-spore isolates were successfully isolated. Among 6 preservation methods tested, periodic transfer and paraffin oil overlay were the best methods that kept the cultures viable for as long as 15 months when the experiment was terminated. There 2 methods have been regularly used by most researchers working with fungi (Fang, 1979; Zhong *et al*, 2005 and Wang *et al*, 2009). Considering the ease of application, periodic transfer should be the method of choice if the experiment does not last too long but if it takes longer to finish, oil overlay should be considered because the fungus will be less active hence lessen the problem of contamination and the fungus loosen its pathogenicity. This experiment has omitted the cold storage because there have been reports showing that *U. virens* could loose its viability or pathogenicity when kept at –20°C (Zhang *et al*., 2003; Liu *et al*., 2007). It is also surprised to find out that rice grain could not be used to preserve *U. virens*. The fungus had lost its viability after being kept for only 2 months. This preservation method was tried because, in nature, the fungus lives and over seasons in rice grain. Therefore, it should have been preserved well in this medium. The opposite result found in this experiment could have come from the difference in morphological form of the fungus under different growing condition. Under the natural condition, most

mycelia formed inside the grain turn into chlamydospores before the smut ball fully mature (Webster and Gunnell, 1992) but under the artificial culturing condition only mycelia and conidia were observed. These 2 forms could have less stability comparing to the chlamydospores to survive the dry-grain condition.

5.2 Among the 7 liquid media tested, potato sucrose broth (PSB) was found to be the most suitable for conidial production of *U. virens.* At 9 days after incubation, it could produce an inoculum at the concentration of 7.25×10^{7} conidia. ml^{-1} which was more than 3 times of what has been reported by Li (2008) using the same medium. In potato dextrose broth (PDB), the second best medium, the spore concentration were only 5.36×10^{6} conidia. ml^{-1}, more than 10 time less compared to that of the PSB. This indicates that sucrose is a better stimulator for conidial production than dextrose at least in *U. virens.* The reason why conidial production was very low in corn broth (CB) could have resulted from its very low sugar content. Sucrose has been reported by many workers to be the best carbon source for conidial production of *U. virens* (Wang, 1992; Ji, 2002). Conidia have been reported to be the most important inoculum for infection under both the natural and artificial conditions (Chen; Xiao and Zhao 1995; Wang *et al.,* 1996). A culture medium that can well support the conidial production is therefore essential for the study of *U. virens*.

5.3 Among the 2 inoculation methods applied at 3 rice growth stages, conidia injection at late booting appeared to be the most effective giving the smut incidence of 50.43% while conidia spraying at the same growth stage gave only 34.75% incidence. Both methods at early booting gave lower smut incidence, while applying inoculum at tillering failed to cause any infection. This finding indicates that growth stage is an important factor contributing to the success or failure of the inoculation. Similar

finding has been reported by Wang *et al.* (1996) who found that the smut incidence was higher when rice plants were injected than when they were sprayed with conidia suspension and the critical susceptible stage was at booting.

5.4 When the pathogenicity was tested among 8 selected *U. virens* isolates on Zhongyou 177 rice cultivar, the 2008-11-1 isolate gave the highest virulence score of 9 with the disease incidence of 81.66% while the 2007-48-1 isolate gave the lowest score of only 5 with the disease incidence of 15.57%. Such results reflected the diversity in virulence among the *U. virens* isolates. The 2008-11-1 isolate was collected from Qiannan which was the major planting area for hybrid rice with excessive use of nitrogen fertilizer, a very conducive condition for *U. virens* epidemic (personal observation,2011). Smut incidence of 15.55% has been commonly observed on rice grown in Qiannan. Crop monoculture and excessive use of nitrogen have been know to cause epidemic of many rice diseases (Ou, 1972; Long, Lee and TeBeest, 2000). The existing of aggressive race in Qiannan should be made known so that the farmers would be more cautious in selecting rice cultivars and applying nitrogen fertilizer.

5.5 The 8 selected *U. virens* isolates were further tested on 6 rice varieties using the conidia injection at late booting stage. Most of the rice varieties tested showed different resistant level from those that had been observed in the field prior to the experiment. Most varieties were either susceptible or moderately susceptible except Fengyouxiangzhan that was highly susceptible to *U. virens* isolate 2007-79-1 and Nongfengyou 256 was moderately resistant to the same *U. virens* isolate. The different reaction observed in the field could have come from the existing of different *U. virens* races in different locations, the phenomenon that has been noted earlier.

After combined analysis, such diversity could be seen again in that among the 8 *U. virens* isolates tested, 2008-33-1 isolate was found to be most aggressive giving disease incidence as high as 58.09% while 2008-2-2 isolate gave only 28.17%. Of all cultivars tested, Gangxiang 827, Jixiangyou 830 and Fengyouxiangzhan had highs smut incidence of 55.06%, 54.79% and 53.50% respectively while Nongfengyou 256 had the lowest of only 23.85%. This cultivar appeared to perform best being moderately resistant to 12.5% of the *U. virens* isolates and moderately susceptible to 87.5% of them. The consistent reaction of this cultivar to *U. virens* observed in the fields with that observed in this experiment indicate that its resistance could be polygenic. The higher disease score observed in the experiment could result from excessive concentration of the inoculum applied to the rice plant and the unnatural inoculation process. With all these shortcomings of the artificial inoculation, it is therefore necessary to repeat the screening under field condition before the varieties could be labeled for their reaction to *U. virens.*

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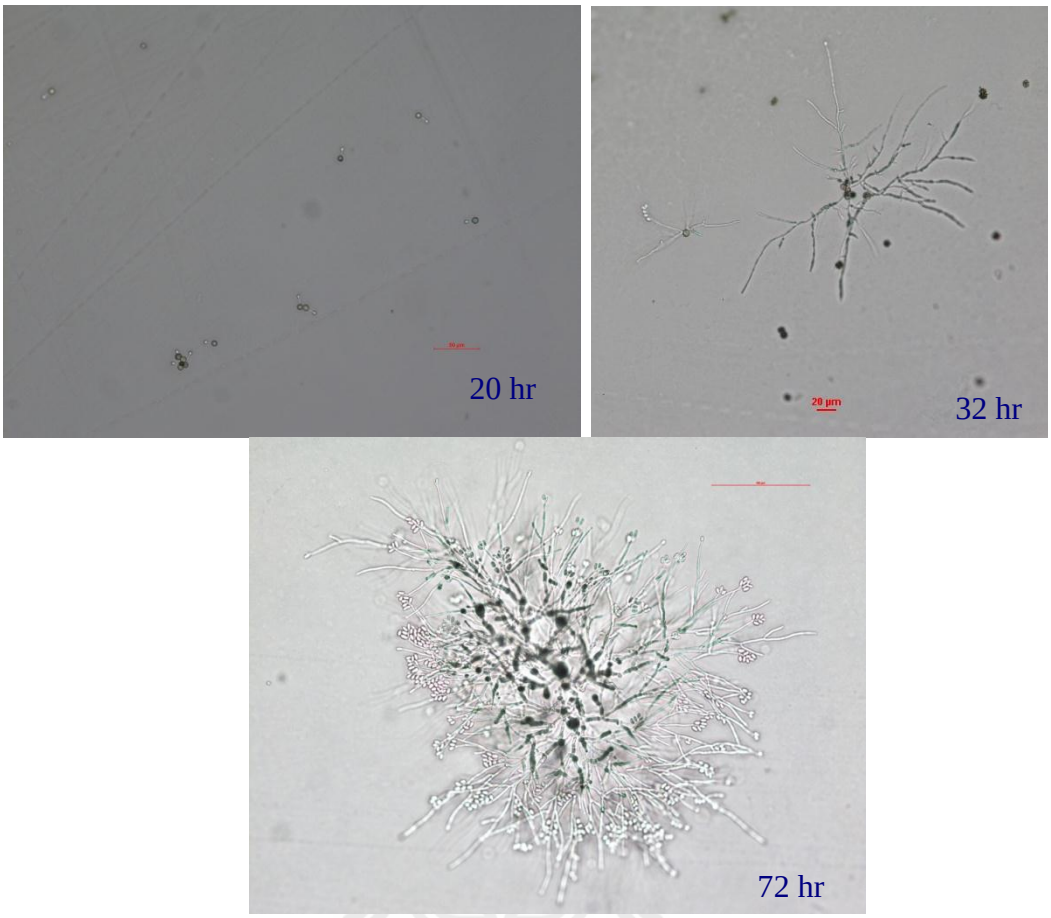
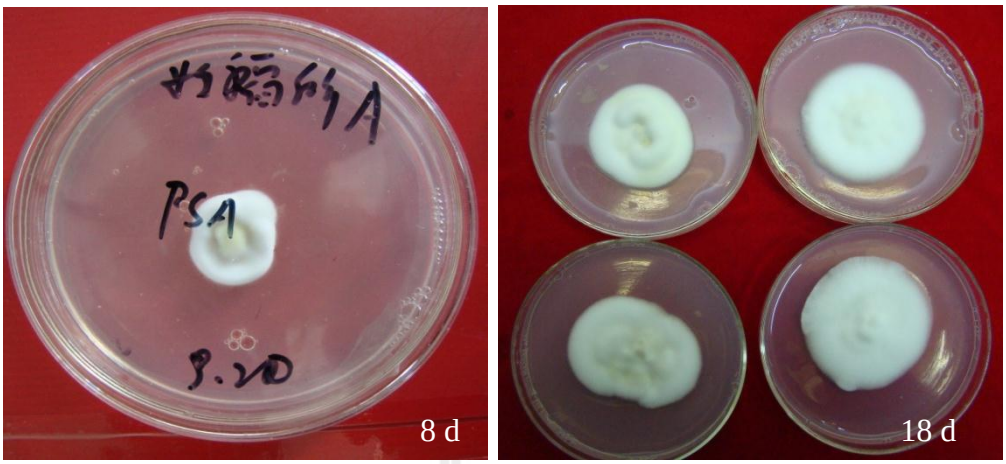
APPENDICES

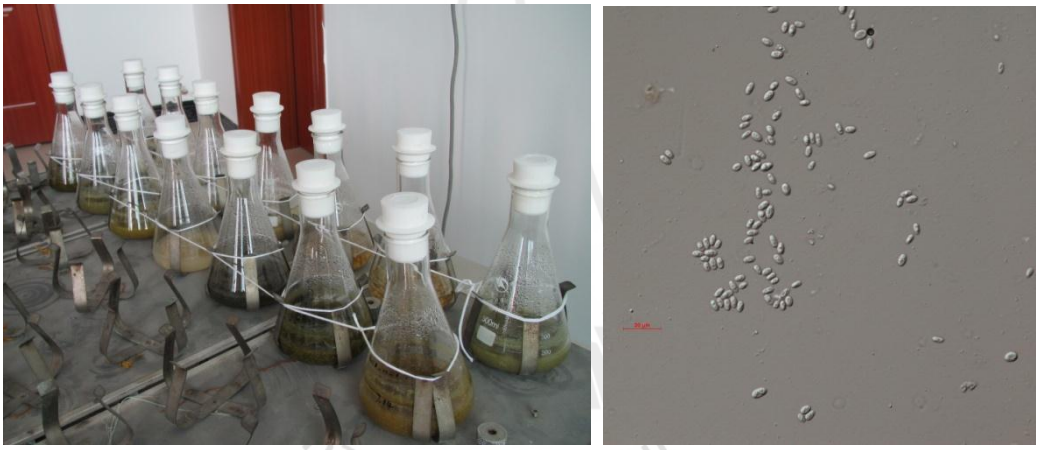


Appendix figure 1 Germinating chlamydospore of *U. virens* at different time

Appendix figure 2 *U. virens* single spore isolates by isolated



Appendix figure 3 Colony of *U. virens* cultured for different period



Appendix figure 4 Artificial shake culture and conidia

Appendix figure 5 Symptoms of *U. virens* in the field

Appendix figure 6 Greenhouse and rice plants of experiments

Appendix figure 7 Sign and symptoms of rice false smut developed at different times after artificial inoculation

BIOGRAPHY

He Haiyong was born on June 23, 1976 in Guizhou province, the People's Republic of China. He earned a bachelor degree in agriculture from the Department of Plant Protection, Agricultural Institute of Guizhou University in 2003. Then he worked at Guizhou Academy of Agricultural Sciences. In 2009, he started to study for a master degree under the supervision of Dr. Sopone Wongkaew at the School of Crop Production Technology, Suranaree University of Technology, Thailand.