การคัดเลือกเชื้อ *Trichoderma* ที่มีประสิทธิภาพในการควบคุมโรคเหี่ยวในมันฝรั่งที่เกิดจากเชื้อ *Verticillium*

นายเฉิน เซียวจุน

วิทยานิพนธ์นี้สำหรับการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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SCREENING FOR EFFECTIVE *Trichoderma* ISOLATES FOR CONTROLLING *Verticillium* WILT IN POTATO

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A Thesis Submitted in Fulfillment of the Requirements for the

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SCREENING FOR EFFECTIVE *Trichoderma* ISOLATES FOR CONTROLLING *Verticillium* WILT IN POTATO

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

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เฉิน เซียวจุน : การคัดเลือกเชื้อ *Trichoderma* ที่มีประสิทธิภาพในการควบคุมโรคเหี่ยวในมันฝรั่งที่เกิดจากเชื้อ *Verticillium* (SCREENING FOR EFFECTIVE *Trichoderma* ISOLATES FOR CONTROLLING *Verticillium* WILT IN POTATO) อาจารย์ที่ปรึกษา : ดร.โสภณ วงศ์แก้ว, 55 หน้า.

วัตถุประสงค์ของงานวิจัยครั้งนี้คือเพื่อคัดหาเชื้อ *Trichoderma* ไอโซเลตที่มีประสิทธิภาพในการควบคุมโรคเหี่ยว ที่เกิดจากเชื้อ *Verticillium* ในมันฝรั่ง ทำการทดลองโดยการแยกเชื้อ *Verticillium dahlia* จำนวน 20 ไอโซเลต จากมันฝรั่งที่เป็นโรคเหี่ยวที่เก็บจาก 6 อำเภอในเขตมณฑลกุ๋ยโจว ประเทศสาธารณรัฐประชาชนจีน นำมาตรวจความสามารถในการก่อโรคกับมันฝรั่ง 2 พันธุ์คือ Favorita และ Hui-2 โดยใช้วิธีจุ่มรากในสารแขวนลอยเชื้อ (root dip inoculation, RDI) และปลูกเชื้อด้วย microsclerotia (microsclerotia inoculation, MI) พบว่าเชื้อทั้ง 20 ไอโซเลตสามารถก่อโรคได้โดยที่ไอโซเลต VGZ-HZ-4 ทำให้มันฝรั่งที่ทดสอบเป็นโรคจำนวนสูงสุด เพื่อเปรียบเทียบกับไอโซเลตอื่น รองลงมาคือไอโซเลต VGZ-SC-1 และ VGZ-XW-1 การวิเคราะห์จำนวนต้นที่เป็นโรคเหี่ยวรวมจากการใช้การปลูกเชื้อทั้ง 2 วิธี จากมันฝรั่งทั้ง 2 พันธุ์ที่ปลูกเชื้อไอโซเลต VGZ-HZ-4 และ VGZ-XW-1 พบว่าวิธี MI ให้จำนวนต้นที่เป็นโรคสูงกว่าวิธี RDI และพันธุ์ Favorita ให้จำนวนต้นที่เป็นโรคสูงกว่าพันธุ์ Hui-2 เมื่อนำเชื้อ *V. dahliae* ทั้ง 2 ไอโซเลตนี้ ไปทดสอบกับเชื้อ *Trichoderma* จำนวน 33 ไอโซเลต เพื่อประเมินความสามารถของเชื้อ *Trichoderma* ในการยับยั้งการเจริญทางเส้นไยของเชื้อ *V. dahliae* บนอาหาร potato dextrose agar โดยเชื้อ 33 ไอโซเลตดังกล่าว จำนวน 21 ไอโซเลต ได้จากการแยกเชื้อจากดินที่เก็บจาก 7 อำเภอของมณฑลกุ๋ยโจว 11 ไอโซเลตได้ จากการแยกเชื้อสปอร์เดี่ยวจากไอโซเลต TGZ-150 ที่เก็บรักษาไว้ที่ Guizhou Institute of Plant Protection (GZIPP) และ 1 ไอโซเลตเป็นเชื้อ TGZ-old-81 ที่เก็บรักษาไว้ที่ GZIPP เช่นกัน ผลการทดสอบพบว่า เชื้อ *Trichoderma* ที่ได้จากการแยกสปอร์เดี่ยวและไอโซเลต TGZ-SC-4 มีประสิทธิภาพในการยับยั้งการเจริญของ *V. dahliae* ได้สูงกว่าเชื้อไอโซเลตอื่น ๆ ที่ทดสอบ และเมื่อนำเชื้อจำนวน 10 ไอโซเลต ที่มีประสิทธิภาพสูงสุดไปทดสอบ การย่อยสลาย microsclerotia ของเชื้อ *V. dahliae* ทั้ง 2 ไอโซเลต พบว่าเชื้อทั้ง 10 ไอโซเลต สามารถย่อยสลาย microsclerotia ได้ เมื่อนำเชื้อ *Trichodema* ทั้ง 33 ไอโซเลตไปทดสอบ ความสามารถในการควบคุมโรคเหี่ยวในสภาพกระถางขนาดเล็ก โดยใช้เชื้อ *V. dahlia*e ไอโซเลต VGZ-HZ-4 กับมันฝรั่งพันธุ์ Favorita โดยปลูกเชื้อด้วยวิธี seed dipping พบว่าเชื้อเกือบทุกไอโซเลต สามารถควบคุมโรคได้ 100% ยกเว้น ไอโซเลต TGZ-CH-4, TGZ-NKY-8, TGZ-TC-4, TGZ-TV-1, TGZ-TV-2 และ TGZ-ZY-2 ซึ่งควบคุมโรคได้เพียง 50-75% เมื่อนำเชื้อ *Trichoderma* 4 ไอโซเลต ที่มีประสิทธิภาพสูงสุดคือ ไอโซเลต TGZ-SC-4, TG2-old-81, TGZ-150-5 และ TGZ-ZY-4 ที่อัตราการใช้ 4 ระดับไปทดสอบความสามารถ

ในการควบคุมโรคเหี่ยวจากเชื้อ *V. dahliae* ไอโซเลต VGZ-HZ-4 ในสภาพเรือนทดลอง โดยใช้มันฝรั่งพันธุ์ Favorita และวิธีปลูกเชื้อวิธีเดียวกับการทดลองในสภาพกระถางเล็ก พบว่าทุกไอโซเลตสามารถควบคุมโรคได้ 100% ในทุกอัตราที่ใช้เมื่อใส่เชื้อ *Trichoderma* ให้กับดินก่อนปลูกมันฝรั่งที่ปลูกเชื้อ *V. dahliae* แต่เมื่อให้เชื้อ *Trichoderma* หลังจากปลูกหัวมันฝรั่งติดเชื้อพบว่า เฉพาะไอโซเลต TGZ-150-5 และ TGZ-ZY-4 เท่านั้น ที่ควบคุมโรคได้ในระดับ 40% ที่อัตราเชื้อ 20 กรัมต่อดินปลูก 1 กิโลกรัม ขณะที่เชื้อ 2 ไอโซเลต ที่เหลือควบคุมโรคได้เพียง 20% ในอัตราเดียวกัน การเพิ่มอัตราของเชื้อ 2 ไอโซเลตแรกมีผลทำให้ต้นพืชรอดตายมีจำนวนลดลง

สาขาวิชาเทคโนโลยีการผลิตพืช
ปีการศึกษา 2555

ลายมือชื่อนักศึกษา_____
ลายมือชื่ออาจารย์ที่ปรึกษา_____
ลายมือชื่ออาจารย์ที่ปรึกษาร่วม_____

CHEN XIAOJUN : SCREENING FOR EFFECTIVE *Trichoderma* ISOLATES FOR CONTROLLING *Verticillium* WILT IN POTATO. THESIS ADVISOR : SOPONE WONGKAEW, Ph.D., 55 PP.

BIOLOCICAL CONTROL/POTATO *VERTICILLIUM* WILT/*TRICHODERMA*

Objective of this research was to screen for effective *Trichoderma* isolates to control *Verticillium* wilt in potato. Twenty isolates of *Verticillium dahliae* were isolated from wilted potato specimens collected from 6 districts in Guizhou, China. All isolates were evaluated for pathogenicity on 2 potato cultivars, Favorita and Hui-2 using the root dip inoculation (RDI) and microsclerotia inoculation (MI) methods. All of the *V. dahliae* isolates appeared to be pathogenic with VGZ-HZ-4 isolate giving the highest wilt incidence compared to the others, followed by VGZ-SC-1 and VGZ-XW-1 isolates. The wilt incidences of the 2 inoculation methods and the two potato cultivars for VGZ-HZ-4 and VGZ-XW-1 isolates indicated that the MI method gave higher wilt incidence than that of the RDI method and the Favorita cultivar had higher wilt incidence than that of Hui-2 cultivar. These 2 *V. dahliae* isolates were further used as representative isolates for mycelial inhibition (MyI) test with 33 *Trichoderma* isolates under dual culture conditions on potato dextrose agar plate. The 33 *Trichoderma* isolates consisting of 21 isolates isolated from potato soils from 7 districts of Guizhou, 11 isolates from single spore isolation of the TGZ-150 isolate preserved at Guizhou Institute of Plant Protection (GZIPP) and 1 isolate TGZ-OLD-81 also preserved at GZIPP. Most of the single spore isolates and TGZ-SC-4 were found to have higher MyI efficiency than that of the rest. Ten of these isolates with high MyI efficiency could completely disintegrate microsclerotia of the 2 *V. dahliae*

isolates. All 33 *Trichoderma* isolates when subjected to small pot screening for wilt controlling efficacy using Favorita as a test variety, *V. dahliae* VGZ-HZ-4 as a test isolate and seed dipping as inoculation technique, could control the disease at 100% efficacy except isolates TGZ-CH-4, TGZ-NKY-8, TGZ-TC-4, TGZ-TV-1, TGZ-TV-2 and TGZ-ZY-2 that had only 50-75% efficacy. The 4 best *Trichoderma* isolates, TGZ-SC-4, TGZ-OLD-81, TGZ-150-5 and TGZ-ZY-4 at different dosages were further tested for efficacy in controlling wilt from *V. dahliae* VGZ-HZ-4 under greenhouse conditions using the same potato test variety and inoculation technique as in the small pot test. Most of the test isolates showed 100% disease controlling efficacy regardless of the dosages if applied before *V. dahliae* inoculation but when they were applied after the *V. dahliae* inoculation, TGZ-150-5 and TGZ-ZY-4 could control the wilt at 40% efficacy at 20 gm.kg soil^{-1} while the other 2 isolates had only 20% efficacy. Increasing dosages of these 2 isolates appeared to reduce the number of survived plants.

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LIST OF ABBREVIATIONS

ANOVA	=	Analysis of variance
APDA	=	Acidified potato-dextrose agar
CMDA	=	Cornmeal dextrose agar
CRD	=	Complete randomized design
GZAAS	=	Guizhou Academy of Agricultural Sciences
GZIPP	=	Guizhou Institute of Plant Protection
MD	=	Microsclerotia disintegration
MDA	=	Microsclertia disintegration ability
MDSI	=	Mean disease severity index
MG	=	Microsclerotia germination
MI	=	Microsclerotia inoculation
MPD	=	Microsclerotia paper discs
MyI	=	Mycelial inhibition
PDA	=	Potato dextrose agar
RDI	=	Root dip inoculation
TSM	=	*Trichoderma*-selective medium

CHAPTER I

INTRODUCTION

1. 1 General introduction

Potato (*Solanum tuberosum* L.) is the World's and China's fourth largest staple crop after rice, wheat and maize (Qu *et al.*, 2005). China is the largest potato producer worldwide, accounting for 26.3% and 22.2% of the global total area and yield (Wang *et al.*,2011). Potato is widely grown in China, from south (Hainan) to north (Inner Mongolia) and from east (Zhejiang) to west (Tibet). Four major potato zones (Fig. 1.1) are distinguished according to the climatic conditions and the cropping systems (Xie, 2007; Jansky *et al.*, 2009; Li and Feng, 2009).

Figure 1.1 Sketch map of four major potato-growing zones in China.

Zone I : northern single-crop zone;

Zone II : central double-crop zone;

Zone III : southern winter-crop zone and

Zone IV: southwestern mix-cropping zone (Wang *et al*.,2011).

Guizhou is not only the largest potato producer in China, but also is potato production base in south China. It has been planned an advantage region for potato planting by Ministry of Agriculture of China, including, Weining, Shuicheng, Bijie, Changshun, and Xiuwen. The planted area in 2010 was more than 733,400 hectares with the production of 2.226 million tons, total output value was over 10 billion CNY (Guizhou yearbook, 2011). So potato is an important economic and food crop in Guizhou. However, the potato yield and quality has been seriously influenced by various diseases. Among problems of potato, *Verticillium* wilt is one of the most destructive diseases that occurs in major potato growing regions worldwide and has been reported in many countries, like USA , New Zealand, the Pacific North-West, Canada and China (Rowe and Powelson, 2002; Mpofu and Hall, 2002; http://www.crop.cri.nz/home/products-services/publications/broadsheets/126-potato.pdf). *Verticillium* wilt is a complex disease caused by many species of *Verticillium*, such as *V. dahliae* Kleb or *V. alboatrum* (Francl *et al.*, 1987; Rowe and Powelson, 2002). However, it is mainly caused by *V. dahliae* which can cause wilt , severe yield and quality losses in more than 160 plant species, such as potato (*Solanum tuberosum* L.), cotton (*Gossypium* spp.), tomato (*Lycopersicon esculentum* Mill.), alfalfa (*Medicago sativa* L.), strawberry (*Fragaria grandiflora* Ehrh.), mint (*Mentha piperita* L.), sunflower (*Helianthus annuus* L.) and eggplant (*Solanum melongena* L.) (Gazendam, Oelofse and Berger, 2004; Uppal, Hadrami and Adam, 2008; Cirulli, 1981; http://www.crop.cri.nz/home/products-services/publications/broadsheets/126-potato.pdf). This disease continues to have a considerable impact on the potato industry, worth about $ US 44 million annually (Mpofu and Hall, 2002**)** and *Verticillium* spp. were present over 60% of potato fields in the U.S.A. (Slattery and

Eide, 1980). Furthermore, it can cause total loss in individual field. *V. dahliae* is one of the most destructive soil/seed-borne fungal pathogen (Uppal, Hadrami and Adam, 2008) and can survive in the soil for 5-14 years (http://www.crop.cri.nz/home/ products-services/publi-cations/broadsheets/126-Potato.pdf). So management of *Verticillium* wilt is challenging due not only to the endogenous growth of the pathogen, but also to its ability to infect multiple hosts and to the multi-year longevity of its propagules in the soil (AlstrÖm, 2001).

The soil-borne pathogens like *Verticillium* spp. are difficult to control, even by chemicals. One reason could be the complicated ecosystem of the soil, where a number of interactions occur. Under favorable conditions such diseases spread rapidly, almost without any possibility of control by fungicides (Galletti *et al.*, 2008). Although, the agricultural chemical fungicides have been used for so long and the effects were prominent, they could induce the pathogen to develop resistance. Moreover, although the disease had been so controlled, some beneficial microbes have also been killed, thus disturbing the ecological balance. There is now a need for other methods of control as fungicidal use may be limited in the future by governmental regulations (Kexiang, Xiaoguang and Yonghong, 2002).

In recent years, sustainable agricultural systems aimed at safeguarding the environment have gained more and more interest, and considerable efforts have been made to adopt strategies which reduce chemical inputs. Less aggressive alternatives are represented by biological methods but the awareness of their moderate effectiveness suggests combining some of them in a multiple integrated approach (Gamliel, Austerweil and Kritzman, 2000). With the increased concern about conserving natural resources as air, soil and water, natural or biological control of

plant diseases has received increased emphasis. Biological control of plant disease is slow, gives few quick profits, but can be long lasting, inexpensive and harmless to life (Zamanian, Shahidi Bonjar and Saadoun, 2005).

Trichoderma species as biocontrol agents of plant pathogen were first recognized in the early 1930's and subsequently they were applied successfully as biocontrol agents against several plant diseases in commercial agriculture (Hjeljord and Tronsmo, 1998; Harman, 2006; Schubert and Fink, 2008). *Trichoderma* is a genus which include species of free-living soil fungi, opportunistic, avirulent plant symbionts (Harman *et al.*, 2004), asymptomatic endophytes (Williama *et al.*, 2003), and parasites of other fungi (Harman, 2006). It is often the major component of the microflora in soils of various ecosystems, such as agricultural farm soil, grassland, forest, marshes, deserts and water (Danielson and Davey, 1973). It possess high reproductive capacity, ability to survive under very unfavorable conditions, efficiency in the utilization of nutrients, and capability to modify the rhizosphere. They produce a variety of compounds that induce localized or systemic resistance responses in plants (Woo *et al.*, 2005)..

In potato, it has been successfully applied to control diseases, such as, stem canker and black scurf (*Rhizoctonia solani*). *T. harzianum*, *T. viride*, *Bacillus subtilis*, and others have been identified to be effective against *R. solani* and dry rot *(Fusarium* spp.) (Kaur and Mukerji, 2004). However, up to date, there has been no report of using *Trichoderma* spp. to control *Verticillium* wilt in potato.

1. 2 Objective of the study

To screen for effective *Trichoderma* isolates for controlling *Verticillium* wilt in potato.

1. 3 Scope of the study

The experiments were conducted both under laboratory and pot condition using the *Trichoderma* isolates from Guizhou and the *Verticillium dahliae* isolates from Guizhou.

1. 4 Anticipated outcomes

1. Obtaining *Trichoderma* isolates that can effectively control *Verticillium* wilt in potato.

2. Obtaining a protocol for testing *Trichoderma* efficacy in controlling *Verticillium* wilt in potato

CHAPTER II

LITERATURE REVIEWS

2.1. *Verticillium* wilt of potato

2.1.1 Occurrence and importance

Verticillium wilt of potato mainly caused by *V. dahliae* Kleb is one of the most economically important and widespread diseases of the cultivated potato. It has a great impact on both the potato production and potato industry. Production reduction over 60% in U.S.A. (Slattery and Eide, 1980) worth about $44 million annually have been reported (Mpofu and Hall, 2002). *Verticillium* wilt of potato in China was first found at Changshun, Guizhou in 2000, but now, has spread to many areas of Guizhou, like Wei ning, Shuicheng, Bijie, Changshun, and Xiuwen (Zhang, 2004).

2.1.2 The symptoms

Foliar symptoms first appear as chlorosis and necrosis beginning at the lower leaves. On warm, sunny days, leaves may appear limp and flaccid. Sometimes symptoms occur on only one side of the leaf or the plant (Fig. 2.1). In severely diseased plants, medium-tan discoloration of the vascular tissue is evident (Fig. 2.1), and the plants may be stunted. When a cross section of an infected tuber is cut from the stem end, tubers of some cultivars may develop a light brown discoloration of the vascular ring (Fig. 2.2), although the presence of the discolored vascular ring is an important diagnostic characteristic, this symptom is associated with other diseases and physiological factors. *Verticillium* spp. do not cause tuber rot. However, some potato

varieties may turn pink around eyes or show pinkish brown blotches on other parts of the tuber when infected with *Verticillium* spp. Pinkeye is frequently associated with *Verticillium* wilt, but is not a *Verticillium* wilt symptom. Tuber yield is reduced because of the decreased rate of photosynthesis and premature death of foliage. The optimum temperature range for potato growth is 18-20°C (64-68°F). When the temperature rises above 20°C (68°F), plant stress increases and symptoms of *Verticillium* wilt are more severe (Berlanger and Powelson, 2000; Johnson, http://www.umaine.edu/umext/potatoprogram/Fact%20Sheets/*Verticillium*%20wilt.pdf)

Figure 2.1 Symptoms of *Verticillium* wilt in potato, unilateral leaf necrosis and wilt in potato

(A) longitudinal section of a potato stem with vascular discoloration

(B) compared to non-affected stem

(C) (Berlanger and Powelson, 2000).

Figure 2.2 *Verticillium* wilt with discoloration of tuber vascular tissue.
(http://pubs.cas.psu.edu/freepubs /pdfs/agrs75.pdf;
http://www.umaine.edu/umext/potatoprogram/Fact%20Sheets/*Verticillium*
%20wilt.pdf)

2.1.3 Disease cycle

The *Verticillium* fungus overwinters in the soil and plant debris as dormant mycelium or black, speck-sized bodies (microsclerotia). Those bodies remain viable for many years. When suitable conditions occur, these microsclerotia germinate by putting forth one or more threadlike hyphae. These hyphae may penetrate the root hairs directly, but more infection is aided by breaks or wounds in rootlets caused by insects, cultivating or transplanting equipment, frost injury, or root-feeding nematodes.

Once inside the root, the fungus invades the water-conducting tissue (xylem). Spread of the fungus into the aerial parts of the plant may be hastened by movement of spores (conidia) in the transpiration stream. These conidia become lodged in the

vascular tissue where they germinate and produce small, mycelial mats. These mats, in turn, produce more conidia which are then carried upward. Runner plants may become infected by movement of the fungus into stolons from the diseased mother plant. Older mycelia produce microsclerotia in host tissues, completing the disease cycle (Fig. 2.3).

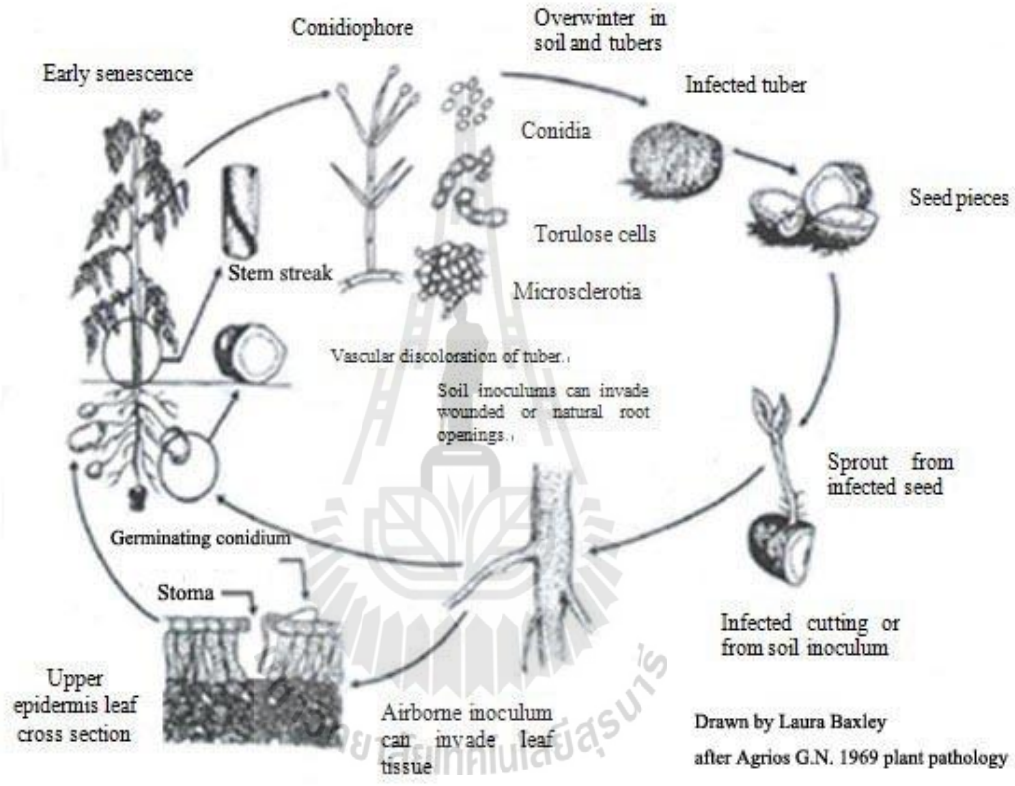

Figure 2.3 Disease cycle of *Verticillium* wilt in potato (http://www.umaine.edu/umext/potatoprogram/Fact%20Sheets/*Verticillium*%20wilt.pdf)

2.1.4 Taxonomic classification

At present, the telemorph of *V. dahliae* is unknown therefore only the anamorph of this fungus is classified as follows

Form-class-Deuteromycetes

Form-order-Moniliales

Form-family-Tuberculariaceae

Genus: *Verticillium*

Species: *Verticillium dahliae*

(http://en.wikipedia.org/wiki/*Verticillium_dahliae*)

2.1.5 General characteristics

Followings are detailed characteristics of *V. dahliae* as described by Shang and Gikim (2012). Because of different strains of *V. dahliae*, two types of characteristics of macrostructure were exhibited at 25 °C after being cultured for two weeks on PDA. Type A, the colonies were pure white and relatively thin. The colony texture was velvety with a little toothed margin. On the surface of colonies, numerous microsclerotia were produced. The reverse of colonies was black in the middle and white at the edge. Type B, the colonies were pure white, thick and turbid. The texture of colony margin was smooth. There was no microsclerotia formed on the surface of colony. The reverse of colonies were pure white on PDA media. For the Characteristics of microstructure, conidiophores were hyaline, simple or branched. Branching of the conidiophores occurred in whorls, verticillate at several levels and the conidia were formed on the top of conidiophores (Fig. 2.4). Hyaline hypha was very long and straight, with the length of 15-25 μm. Microsclerotia were formed by

numerous irregular cycloid (Fig. 2.4C). The size of microsclerotia were big enough to be observed with naked eye，nearly 25-75× 0-65 μm with black color. Conidia were hyaline or brightly colored, 2.5-4×1-1.5 μm in size，one-celled，and oval to pyriform in shape.

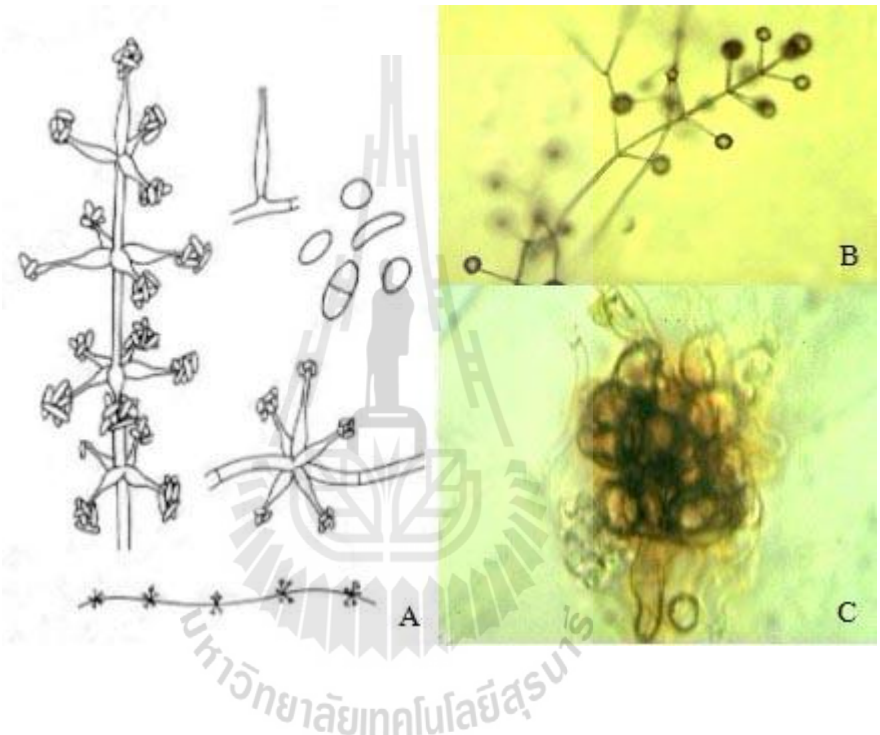

Figure 2.4 Characteristics of conidiospore (A), conidiophores (B), and microsclerotia (C) of *V. dahliae*
(http://www.cals.ncsu. edu/course/pp728/*Verticillium*/Vertifin.htm)

2.2 Control of *Verticillium* wilt in potato

Verticillium propagules occur in highest concentration in the top 30 cm (12 inches) of the soil profile, but they have been recovered from depths as low as 41 cm (16 inches). Inoculum densities and disease severity tend to increase from year to year

if crops are planted. So, it is difficult to control *Verticillium.* Management practices are aimed at reducing the populations of these pathogens in soil or changing the susceptibility of the host. Potential components of integrated systems are cultural methods (crop rotation, green manures, fertilization, irrigation, vine removal), host resistance, fungicides (primarily fumigation), and biological controls (http://www.crop.cri.nz/home/products-ervices/publications/broadsheets/126-Potato. pdf). Soil fumigation may be used to reduce high levels of soilborne inoculum. Methyl bromide, metam, chloropicrin or dazomet can be used to control *Verticillium*, but such control (Uppal, Hadrami and Adam, 2008), is costly, noxious, and not always sufficient to reduce the inoculum concentration in the soil. Therefore, other methods, such as cultural practices, solarization, and the use of tolerant cultivars, are better alternatives. The most common rotation in Ontario is potato-rye/corn-potato. Such short rotations of two to three years have not reduced the population of *V. dahliae* (Mpofu and Hall, 2002).

2.3 Biological control of *Verticillium* wilt by *Trichoderma* spp.

2.3.1 General characteristics of *Trichoderma* spp.

Followings are the characteristics of *Trichoderma* spp. as described in wikipedia (http://en.wikipedia.org/wiki/*Trichoderma*). Cultures are typically fast growing at 25-30°C, but will not grow at 35°C. Colonies are transparent at first on media such as cornmeal dextrose agar (CMDA) or white on richer media such as potato dextrose agar (PDA). Mycelia are not typically obvious on CMDA, conidia typically form within one week in compact or loose tufts in shades of green or yellow or less frequently white. A yellow pigment may be secreted into the agar, especially on

PDA. Some species produce a characteristic sweet or ‘coconut’ odor. Conidiophores are highly branched and thus difficult to define or measure, loosely or compactly tufted, often formed in distinct concentric rings or borne along the scant aerial hyphae. Main branches of the conidiophores produce lateral side branches that may be paired or not, the longest branches distant from the tip and often phialides arising directly from the main axis near the tip. The branches may rebranch, with the secondary branches often paired and longest secondary branches being closest to the main axis. All primary and secondary branches arise at or near 90° with respect to the main axis. The typical *Trichoderma* conidiophore, with paired branches assumes a pyramidal aspect. Typically the conidiophore terminates in one or a few phialides. In some species (e.g. *T. polysporum*) the main branches are terminated by long, simple or branched, hooked, straight or sinuous, septate, thin-walled, sterile or terminally fertile elongations. The main axis may be the same width as the base of the phialide or it may be much wider (Fig. 2.5A and 2.5B). Phialides are typically enlarged in the middle but may be cylindrical or nearly subglobose. Phialides may be held in whorls, at an angle of 90° with respect to other members of the whorl, or they may be variously penicillate (gliocladium-like). Phialides may be densely clustered on wide main axis (e.g. *T. polysporum, T. hamatum*) or they may be solitary (e.g. *T. longibrachiatum*). Conidia typically appear dry but in some species they may be held in drops of clear green or yellow liquid (e.g. *T. virens, T. flavofuscum*). Conidia of most species are ellipsoidal, 3-5×2-4 µm (L/W≥1.3); globose conidia (L/W<1.3) are rare. Conidia are typically smooth but tuberculate to finely warted conidia are known in a few species. Synanamorphs are formed by some species that also have typical *Trichoderma* pustules. Synanamorphs are recognized by their solitary conidiophores

that are verticillately branched and that bear conidia in a drop of clear green liquid at the tip of each phialide. Chlamydospores may be produced by all species, but not all species produce chlamydospores on CMD at 20°C within 10 days. Chlamydospores are typically unicellular subglobose and terminate short hyphae; they may also be formed within hyphal cells. Chlamydospores of some species are multicellular (e.g. *T. stromaticum*)

Figure 2.5 Conidia, Conidiophores (A) (http://en.wikipedia.org/wiki/*Trichoderma*) and colony characteristics (B) of *T. harzianum* TGZ-150 on PDA

2.3.2 *Trichoderma* as a biological control agent

Trichoderma is a genus of fungi that is present in all soils, where they are the most prevalent culturable fungi. Many species in this genus can be characterized as opportunistic avirulent plant symbionts (Harman *et al.*, 2004). Several strains of *Trichoderma* have been developed as biocontrol agents against fungal diseases of

plants (Harman, 2006). Since Weindling (1932) recognized the antagonistic effect of *Trichoderma* species against plant pathogens, several species of it have been extensively studied as biological control agents against fungal pathogens as well as plant growth enhancers. Commercial products based on selected *Trichoderma* isolates are currently utilized in the biological control of many pathogenic fungi, from soil/seed-borne to foliar pathogens (Monte, 2001; Kubicek, 2001), and mainly applied to the soil such as, eggplant *Verticillium* wilt (*V. dahliae* Kleb) (Narisawa *et al.*, 2002), and there are few reports dealing with their applicacation in the management of aerial diseases (Blakeman and Fokkema, 1982). Moreover, *Trichoderma* species have been utilized to control root diseases caused by nematodes (Sharon *et al.*, 2001; Sahebani and Hadavi, 2008). *Trichoderma* spp. were able to produce diffusible and/or volatile antibiotics, hydrolytic and lytic enzymes like β-1,3-glucanase, cellulase, hemicellulase, xylanase and chitinase. These hydrolytic enzymes partially degrade the pathogen cell wall and leads to its parasitization and directly inhibit pathogen growth (Wong and Saddler, 1992; Tronsmo and Hjeljord, 1998; Nieves *et al.*, 2004; Manjula *et al.*, 2004; Bailey *et al.*, 2008) and have the ability of promoting plant growth and development (Samuels, 2006), and localized resistance (Howell, 2003; Harman *et al.*, 2004). Up to date, there are many successful cases of *Trichoderma* spp. to control diseases. Such as, *Trichoderma viridae* treatment in the form of a seed dip in a spore suspension (10^8 conidia/ml) and soil drenching with a spore suspension were very effective in reducing infection from brown blotch (*Colletotrichum truncatum*) infected seeds of cowpea (Bankole and Adebanjo, 1996). Integration of *T. harzianum* (10^5 spores/ml/g seed) and carboxin (2 g/kg seed) for seed treatment resulted in enhanced seed germination (12.0–14.0%) and grain yields (42.6–72.9%) and reduced wilt

incidence (44.1–60.3%) in field experiment (Dubey, Suresh and Singh, 2007). In the greenhouse, germlings of *T. hamatum* (actively-growing hyphae on bran) and alginate pellets containing biomass of *Rhizoctonia solani*, significantly prevented disease (> 80%), reduced pathogen inoculum (> 75%), and resulted in increases in the population densities of the biocontrol fungi (Lewis, Barksdale and Papavizas, 1990). Applied to soilless mix at a rate of 1.0% (w/w, dry weight), activated VBA–FB (*Trichoderma* and *Gliocladium*) tested reduced damping-off of pepper, eggplant, zinnia, cucumber, and cabbage seedlings after 4 weeks of growth to yield stands comparable to those in the noninfested control (85%) (Lewis and Lumsden, 2001). *T. harzianum* in both seed coated treatments was more effective in decreasing the mean disease severity index (MDSI) on peanut brown root rot *(Fusarium solani)*, increasing the frequency of healthy plants, and boosting yield.

CHAPTER III

MATERIALS AND METHODS

3. 1 Diseased plant tissue collection and pathogen isolation

Ten potato wilt diseased samples were collected from Shuicheng, Hezhang, Changshun, Weining, Guiyang (GZAAS and Xiuwen) in Guizhou Province, China. Subsequently, the samples were washed in running water for 15-20 min, immersed in 1% sodium hypochlorite (NaOCl) for 2-3 min, rinsed with sterile distilled water for 30-45 sec and then dipped in 70% ethyl alcohol for 20-30 sec. Cross sections were then made under an aseptic condition and transferred onto acidified potato-dextrose agar (APDA). The APDA contained 2 ml of 25% lactic acid per liter (Jong-Tae , In-Hee and Hyang-Burm, 2001). The stem sections were incubated for 14-20 days at 22-24^{o}C as reported by Slattery and Eide (1980). After 4 days, the hyphal tip grown out from each piece of tissue of *V. dahliae* was picked and transferred onto the new PDA medium for further experiment.

3.2 Pathogenicity test of *Verticillium dahliae* isolates

3.2.1 Root dip inoculation (RDI)

Twenty of the *Verticillium dahliae* isolates obtained were tested for their pathogenicity in potato seedlings. All cultures were grown on potato dextrose agar (PDA) at 24±2 ^{o}C prior to inoculation. Spore suspensions were prepared from 3-week-old cultures by adding 10 ml of sterile distilled water to each plate and scraping

the cultures with a rubber spatula. Using a haemocytometer, the inoculum concentration was adjusted to 10^7 conidia/ml. Potato seedlings of the Favorita and Hui-2 varieties were used as test plants. Favorita had been observed to be susceptible to *V. dahliae*, while Hui-2 was resistant. These potato seedlings were uprooted and inoculated by using the root-dip technique. Roots were washed with running water, and placed for 60 minutes in conidia suspension. The inoculated seedlings were subsequently transplanted into pots of sterilized soil. Three replications of five plants for each isolate were used. The plants were kept on a room bench at 21-23^{o}C. Daylight was supplemented by fluorescent lamps to provide a 12 hr day length. About 4 weeks after inoculation, Wilt incidence was checked.

3.2.2 Microsclerotia inoculation (MI)

The same 20 isolates of 3.2.1 were used as representative isolates. After microsclerotia having formed on PDA, the whole agar piece was removed from the plate and put in the soil beneath the roots of 5 transplanted potato seedlings of the same varieties used in 3.3.1 in a 20 cm pot, in three replications. The plants were kept under the same condition as in 3.3.1. About 4 weeks after inoculation, Wilt incidence was observed.

The experimental trials were conducted following a three-factors complete randomized design (CRD).

3.3 Isolation of *Trichoderma* from soil and single spore isolation

3.3.1 *Trichoderma*-selective medium (TSM)

The TSM consisted of a basal medium comprising (all amount is per liter) 0.2 g $MgSO_4$ ($7H_2O$), 0.9 g K_2HPO_4, 0.15 g KCl, 1.0 g NH_4NO_3, 3 g glucose, 0.15 g rose

Bengal, and 20 g agar. These constituents were added to 950 ml of distilled water and autoclaved at 121°C. for 15 min. The antimicrobial and fungicidal ingredients (all amount were per liter) were 0.25 g chloramphenicol, 9.0 ml streptomycin stock solution (1% w/v), 0.2 g quintozene, and 1.2 ml propamocarb (772 g of active ingredient per liter), all in 20 ml of sterile distilled water, and the mixtures were added to the cooled basal medium (40–50°C) (Williama *et al.*, 2003).

3.3.2 Soil sample collection and *Trichoderma* isolation

Soil samples were taken from each *Verticillium* wilt diseased field in Shuicheng , Weining , Hezhang , Zunyi and Guiyang of Guizhou Province, China and 10 fields were sampled. Five soil sub samples were taken from the area around the healthy potato roots, pooled and placed in polyethylene bags and stored at 4°C. Ten gram of the sample was suspended in 50 ml of sterile distilled water and incubated for 30 min at 200 rpm in a rotary shaker. Serial dilutions (5×1:9 ml) were then made. Subsequently, 0.1 ml of each dilution was spread on the TSM surface with a glass rod, 2 replications for each dilution and incubated at 28°C. Five to ten *Trichoderma* single colonies were collected from each sample and transferred onto PDA for further study. The isolates were primarily selected according to their differences in colony characters.

3.3.3 Single spore isolates of TGZ-150 *Trichoderma* strain

Trichoderma harzianum TGZ-150 was isolated from rhizosphere soil of tobacco where tobacco root rot (*Fusarium oxysporum*) was serious in Bijie city of Guizhou Province in 1990's preserved at the Plant Pathology Laboratory of Guizhou Institute of Plant Protection (GZIPP).

Each 0.03 ml of TGZ-150 *Trichoderma* spore suspension (10^3conidia/ml) was spread on a PDA plate. The PDA plates were subsequently incubated at 28±2 ^oC for 12 hours and examined frequently under a microscope for germinating spores which were picked and transferred with a sterile cork borer to fresh PDA plates. The single spore isolates were maintained in PDA slant for the future experiment.

3.4 *In vitro* inhibition of *Trichoderma* isolates to *Verticillium dahliae* mycelial growth

3.4.1 Mycelial inhibition (MyI)

Trichoderma isolates used for this study were from 2 origins. One was the 11 single spore isolates of TGZ150, and TGZ-OLD-81 preserved at the Plant Pathology Laboratory of Guizhou Institute of Plant Protection (GZIPP), the others were those isolated from the fields. *T. harzianum* TGZ-OLD-81 was isolated from sclerotia of *Sclerotium rolfsii Sacc* causing southern blight in peppers, used as bait in the soil of pepper field in Guanling town of Guizhou in 1990's.

Two *V. dahliae* isolates with the highest levels of pathogenicity in potato were tested with *Trichoderma* isolates. *Trichoderma* isolates were tested *in vitro* for their ability to grow over *V. dahliae* colony in dual culture. One mycelial disc (5 mm) of each *Trichoderma* isolate and *V. dahliae* were put together in a PDA Petri dish, 6 cm apart. The test was done in three replications (dishes). The *V. dahliae* mycelial growth inhibition rate was calculated at 3 days after incubation.

The percent growth inhibition rate was calculated as follows:

Growth inhibition (%) = [(RCK-RT/RCK) ×100]

Where RCK is the radius of *V. dahliae* isolate, and RT is the radius of *V. dahliae* isolate that cultured with *Trichoderma* in the same Petri dish .

3.4.2 Microsclerotia disintegration (MD)

Because microsclerotia are the main inocula of *V. dahliae* in the soil, their elimination can reduce wilt incidence in the subsequently growing season. Therefore, *Trichoderma* isolates having microsclertia disintegration ability (MDA) would be more desirable. This experiment was aimed at testing the MDA of some selected *Trichoderma* isolates.

Microsclerotia paper discs (MPD) were prepared. A sterile filter paper disc (5mm) was put at the edge of each growing colony of *V. dahliae* in order to collect microsclerotia. Numerous microsclerotia were produced on the paper disc after 1 week.

Ten *Trichoderma* isolates having highly inhibition ability on *V. dahliae* mycelial growth were tested for their ability to disintegrate *V. dahliae* microsclerotia. One mycelial disc (5 mm) of each *Trichoderma* isolate was put in the middle of the PDA plate. After 1 day, 30 MPDs were put at the edge of the growing colony in 3 replications. The MPD was pretested for their sensitivity to surface disinfectant (1% NaOCl and 75% ethyl alcohol) before the actual experiment was conducted. The best condition of MPD treatment was applied to the MPD for this test. After 1 week, the MPD from each treatment was recovered and checked for the viability. All of the MPDs were put in the PDA plates after disinfectant (75% ethyl alcohol 3min and 1% NaOCl 5min) treatment. With this treatment, only viable microsclerotia survived but all *Trichoderma* propagules were killed. Percentage of non-germinating microsclerotia reflected the ability of *Trichoderma* to disintegrate the microsclerotia.

3.5 Small pot screening for *Trichoderma* efficacy

All 33 *Trichoderma* isolates were subjected to small pot screening for wilt controlling efficacy by preparing each of the *Trichoderma* isolate in the wheat medium. And then mix the culture with an autoclaved sand in 1% ratio (w/w) and put in paper cups. The potato seeds of Favorita were grown after being inoculated with *Verticillium* (VGZ-HZ-4) spore suspension (10^6conidia/ml) by seed dipping method, 1 seed/ cup. The experiment was conducted in CRD with 4 replications. The wilt incidence was checked after the treatment without *Trichoderma* inoculation started to show symptoms.

3.6 Efficacy of *Trichoderma* in controlling *Verticillium* wilt in greenhouse

3.6.1 *Trichoderma* application before *Verticillium* inoculation.

The most effective *Trichoderma* isolates were selected from the 4 isolates showing good performance in *in vitro* and small pot screening test to be used as representatives in this trial. The *Trichoderma* isolates were cultured in the wheat medium and used as soil inoculum. Four levels of inoculum at 10, 20, 40 and 80 gram were mixed with 1000 gram dry pasteurized soil and put into pots. Subsequently, 5 pieces of potato seed were sown into each pot in 4 replications.

The *Verticillium* inoculation based on the result of experiment 3.2 was applied to the potato at sowing time. Subsequently the Wilt incidence was observed after the treatment without *Trichoderma* inoculation started to show symptoms. The experiment was done in 10 treatments, using CRD in 4 replications. The *Verticillium* (VGZ-HZ-4) were inoculated by seed dipping as described in 3.5. Apart from

Trichoderma inoculation, the treatments included 3 controls, healthy controls (no *Trichoderma* and no *Verticillium* inoculation), inoculated with *Verticillium* without *Trichoderma* and inoculated with only *Trichoderma* isolate.

3.6.2 *Trichoderma* application after *Verticillium* inoculation

The experiment was conducted in the same manner as in 3.6.1 but the *Trichoderma* isolates were applied 20 days after *Verticillium* had been inoculated.

3.6.3 Data collection and analysis

Data of wilt incidence and severity were collected. The efficacy of *Trichoderma* to control *Verticillium* wilt was calculated by wilt incidence. An asessment of the wilt incidence on individual plants or vascular discoloration of the basal portion of the stems was conducted using the method of Uppal, Hadrami and Adam (2008). The infection percentage was calculated as follows:

$$\text{Wilt incidence}(\%) = [(CL/TL) \times 100],$$

$$\text{Efficicacy}(\%) = [\ (\text{Wilt incidence}_{\text{control}} - \text{Wilt incidence}_{\text{treatment}})/ \text{Wilt incidence}_{\text{control}}] \times 100$$

Where, CL is the number of chlorotic or wilted plants and TL is the total number of potato plants.

The data was analysed by SPSS 16.0 software. To ensure the homogeneity of the variances and the symmetry of the distribution of each variable, data recorded as percentages were arcsine-transformed before ANOVA analysis in this research.

CHAPTER IV

RESULTS

4.1 Isolation of *Verticillium dahliae* and *Trichoderma*

Twenty isolates of *V. dahliae* were isolated from the planting areas of Guizhou Province (Table 4.1). From the soil samples collected from different location in Guizhou, 21 *Trichoderma* isolates were isolated (Table 4.2). Additional 11 isolates were single-spore isolated from the old collection of *Trichoderma harzianum* TGZ-150, a commercial isolate preserved at the plant pathology laboratory of GZIPP. All these isolates and TGZ-OLD-81 were included in the up-coming experiment.

4.2 Pathogenicity test of *Verticillium dahliae* isolates

The 20 isolates of *V. dahliae* were tested for pathogenicity on two potato cultivars by root dip inoculation and microsclerotia inoculation. All of the *V. dahliae* isolates could infect potato cultivar Favorita (Table 4.3). The combined average Wilt incidence of RDI and MI on the cultivar Favorita was higher than cultivar Hui-2 (Table 4.5) which was 39.63% and 8.62% respectively. Table 4.6 shows the combined wilt incidence resulting from the use of 2 inoculation techniques on the 2 cultivars. It appears that the microsclerotia inoculation gave a higher wilt incidence (25.92%) compared to that of the root dip technique. When results of pathogenicity of the 20 *V. dahliae* isolates on the 2 potato cultivar were combined, it appeared that VGZ-HZ-4

gave the highest wilt incidence of 39.71%, seconded by VGZ-SC-1 and VGZ-XW-1 which gave wilt incidence that were not statistically different (37.41% and 34.62% respectively) (Table 4.4). VGZ-HZ-4 and VGZ-XW-1 were selected as representative isolates for further experiment.

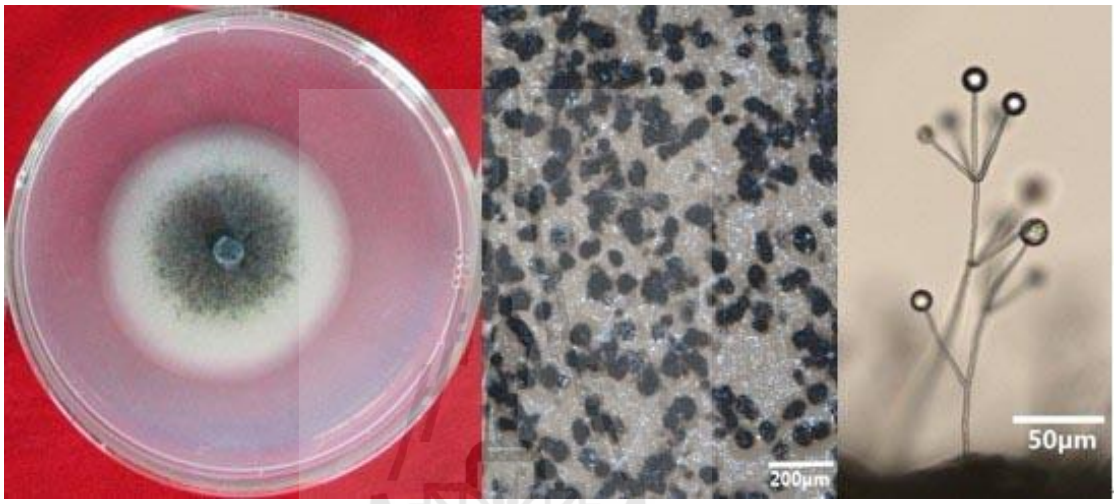

Figure 4.1 Colony character (left), microsclerotia (middle) and conidia (right) of *Verticillium dahliae* on APDA medium.

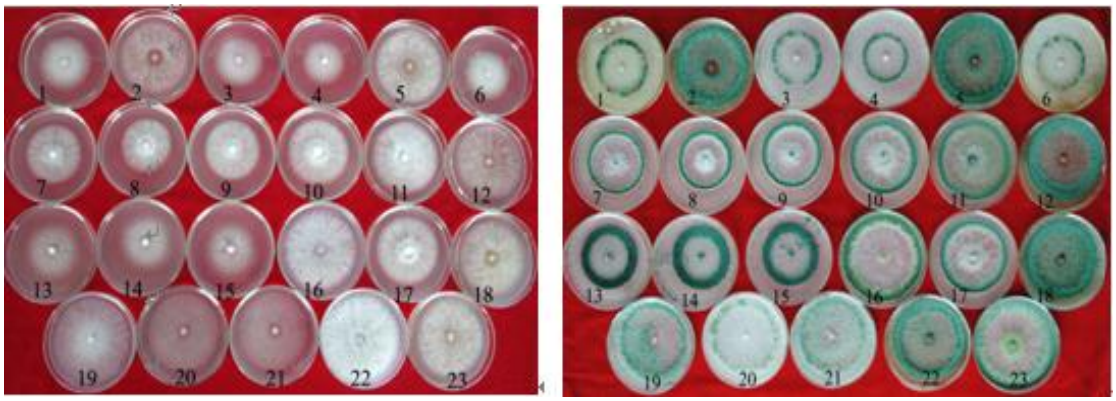

Figure 4.2 The character of *Trichoderma* isolates cultured for 2days (left) and 7 days (right) on PDA. (1 TGZ-TV-1, 2 TGZ-150-37, 3 TGZ-TV-2, 4 TGZ-TV-3, 5 TGZ-150-5, 6 TGZ-150-33, 7 TGZ-CH-1, 8 TGZ-CH-2, 9 TGZ-CH-3, 10 TGZ-CH-4, 11 TGZ-ZY-2, 12 TGZ-ZY-4, 13 TGZ-NKY-1, 14 TGZ-NKY-2, 15 TGZ-NKY-3, 16 TGZ-NKY-5, 17 TGZ-HZ-4, 18 TGZ-OLD-81, 19 TGZ-NKY-7, 20 TGZ-SC-5, 21 TGZ- SC-3, 22 TGZ-SC-4 and 23 TGZ-150-38)

Table 4.1 Isolates of *Verticillium dahliae* isolated from the wilted potato grown in Guizhou Province, China used in the experiment

Isolate	Origin	Host	Isolate	Origin	Host
VGZ-SC-1	Shuicheng	Favorita	VGZ-HZ-1	Hezhang	WeiYu-3
VGZ-SC-2	Shuicheng	Favorita	VGZ-HZ-3	Hezhang	Favorita
VGZ-SC-3	Shuicheng	WeiYu-3	VGZ-HZ-4	Hezhang	Favorita
VGZ-SC-4	Shuicheng	WeiYu-3	VGZ-HZ-9	Hezhang	Favorita
VGZ-SC-5	Shuicheng	WeiYu-3	VGZ-HZ-10	Hezhang	Favorita
VGZ-SC-6	Shuicheng	Favorita	VGZ-XW-1	Xiuwen	WeiYu-3
VGZ-SC-7	Shuicheng	Favorita	VGZ-WN-1	Weining	Favorita
VGZ-CS-1	Changshun	Favorita	VGZ-WN-2	Weining	WeiYu-3
VGZ-CS-2	Changshun	Favorita	VGZ-NKY-2	GZAAS[1]	Favorita
VGZ-CS-5	Changshun	Favorita	VGZ-NKY-4	GZAAS	Favorita

[1] GZAAS: Guizhou Academy of Agricultural Sciences

Table 4.2 *Trichoderma* isolates from the collection and isolated from the soil

Isolate	Origin	Isolate	Origin
TGZ-SC-3	Shuicheng	TGZ-CH-1	Weining
TGZ-SC-4	Shuicheng	TGZ-CH-2	Weining
TGZ-SC-5	Shuicheng	TGZ-CH-3	Weining
TGZ-HZ-3	Hezhang	TGZ-CH-4	Weining
TGZ-NKY-1	GZAAS	TGZ-OLD-81[1]	Guanling
TGZ- NKY -2	GZAAS		
TGZ- NKY -3	GZAAS	TGZ-150-1	Single spore isolate of TGZ-150[2]
TGZ- NKY -5	GZAAS	TGZ-150-4	
TGZ- NKY -7	GZAAS	TGZ-150-5	
TGZ- NKY -8	GZAAS	TGZ-150-6	
TGZ-TC-3	Changshun	TGZ-150-33	
TGZ-TC-4	Changshun	TGZ-150-37	
TGZ-TV-1	Xingyi	TGZ-150-38	
TGZ-TV-2	Xingyi	TGZ-150-41	
TGZ-TV-3	Xingyi	TGZ-150-50	
TGZ-ZY-2	Zunyi	TGZ-150-51	
TGZ-ZY-4	Zunyi	TGZ-150-55	

[1] TGZ-OLD-81 was isolated by baiting from the soil of a pepper field.
[2] TGZ-150 was isolated from rhizosphere of tobacco roots and preserved at GZIPP.

Table 4.3 Wilt incidence of potato cultivars Favorita and Hui-2 inoculated with *Verticillium dahliae* isolates by root dip inoculation (RDI) and microsclerotia inoculation (MI)

Treatment	Wilt incidence (%)			
	Favorita		Hui-2	
	RDI[1]	MI	RDI	MI
VGZ-SC-1	26.67ab	33.33bcd	0.00a	6.67ab
VGZ-SC-2	40.00ab	40.00abcd	6.67a	6.67ab
VGZ-SC-3	46.67ab	33.33bcd	0.00a	0.00b
VGZ-SC-4	33.33ab	40.00abcd	13.33a	13.33ab
VGZ-SC-5	46.67ab	46.67abcd	0.00a	6.67ab
VGZ-SC-6	20.00bc	46.67abcd	6.67a	6.67ab
VGZ-SC-7	46.67ab	53.33abc	0.00a	0.00b
VGZ-HZ-1	20.00 bc	6.67ef	0.00a	6.67ab
VGZ-HZ-3	53.33 ab	46.67abcd	6.67a	0.00b
VGZ-HZ-4	60.00 a	73.33a	20.00a	20.00a
VGZ-HZ-9	20.00 bc	20.00de	0.00a	0.00b
VGZ-HZ-10	40.00ab	66.67ab	6.67a	0.00b
VGZ-CS-1	66.67a	73.33a	13.33a	13.33ab
VGZ-CS-2	40.00ab	53.33abc	0.00a	6.67ab
VGZ-CS-5	33.33ab	26.67cd	0.00a	13.33ab
VGZ-WN-1	40.00ab	53.33abc	13.33a	13.33ab
VGZ_WN-2	40.00ab	60.00abc	6.67a	6.67ab
VGZ-NKY-2	46.67ab	46.67abcd	6.67a	13.33ab
VGZ-NKY-4	20.00bc	40.00abcd	0.00a	0.00b
VGZ-XW-1	46.67ab	60.00abc	13.33a	20.00a
Control[2]	0.00c	6.67f	0.00a	0.00b
F-test	**	**	NS	*
CV (%)	27.23	20.43	35.95	30.54

[1] Means in the same column followed by different letters are statistically different at P≤0.05 by DMRT.

[2] Control: without *Verticillium*

Table 4.4 Combined wilt incidence of Favorita and Hui-2 potato cultivars inoculated by root dip technique and microsclerotia inoculation with different *Verticillium dahliae* isolates

***V. dahliae* isolates**	**Wilt incidence (%)[1]**
VGZ-HZ-4	39.71a
VGZ-CS-1	37.41ab
VGZ-XW-1	34.62ab
VGZ-WN-1	30.30abcd
VGZ-NKY-2	28.18abcd
VGZ-SC-4	27.42abcd
VGZ-WN-2	26.93bcde
VGZ-HZ-10	25.77cde
VGZ-HZ-3	24.81cde
VGZ-SC-2	23.95cde
VGZ-CS-2	23.75cde
VGZ-SC-5	23.66cde
VGZ-SC-7	22.50de
VGZ-SC-6	20.68def
VGZ-SC-3	19.52def
VGZ-SC-1	18.66def
VGZ-CS-5	18.47def
VGZ-NKY-4	15.29ef
VGZ-HZ-9	10.97fg
VGZ-HZ-1	9.91fg
F-test	**
CV (%)	19.81

[1] Means in the same column followed by different letters are statistically different at P≤0.05 by DMRT.

Table 4.5 Combined wilt incidence on 2 potato cultivars inoculated with *Verticillium dahliae* by 2 methods

Potato cultivar	**Wilt incidence(%)[1]**
Favorita	39.63a
Hui-2	8.62b
F-test	**
CV (%)	19.81

[1] Means in the same column followed by different letter are statistically different at P≤0.05 by DMRT.

Table 4.6 Combined wilt incidence of 2 inoculation technique on 2 potato cultivars

Inoculation method	**Wilt incidence[1]**
Microsclerotia inoculation(MI)	25.92a
Root dip inoculation (RDI)	22.31b
F-test	*
CV (%)	19.81

[1] Means in the same column followed by different letters are statistically different at P≤0.05 by DMRT.

4. 3 In *vitro* inhibition of *Trichoderma* isolates on *Verticillium dahliae* mycelia

In this experiment, the 33 *Trichoderma* isolates were tested for mycelial inhibition of the two *V. dahliae* isolates, VGZ-HZ-4 and VGZ-XW-1.

All of *Trichoderma* isolates could grow and occupy the whole colony of *V. dahliae* within 4 days (Fig. 4.3). Most of the *Trichoderma* isolates grew so fast and had the averaged colony radius of 41.86 mm, 54.08 mm and 65.98 mm after 2, 3 and 4 days respectively. After 7 days, all of them could produce spores at the average of 1.38×10^{10}cfu/dish.

After 3 days of dual culture at 28°C, most of the isolates had performed noticeably well on inhibition ability. The inhibition percentage measured at 3 days

after the dual culture is shown in Table 4.7. It can be seen that most of single spore-isolates of TGZ-150 and the TGZ-OLD-81 had higher inhibition efficacy than the average but the one isolated from soil of Shuicheng, TGZ-SC-4 isolate gave the highest inhibition percentage of 38.37%.

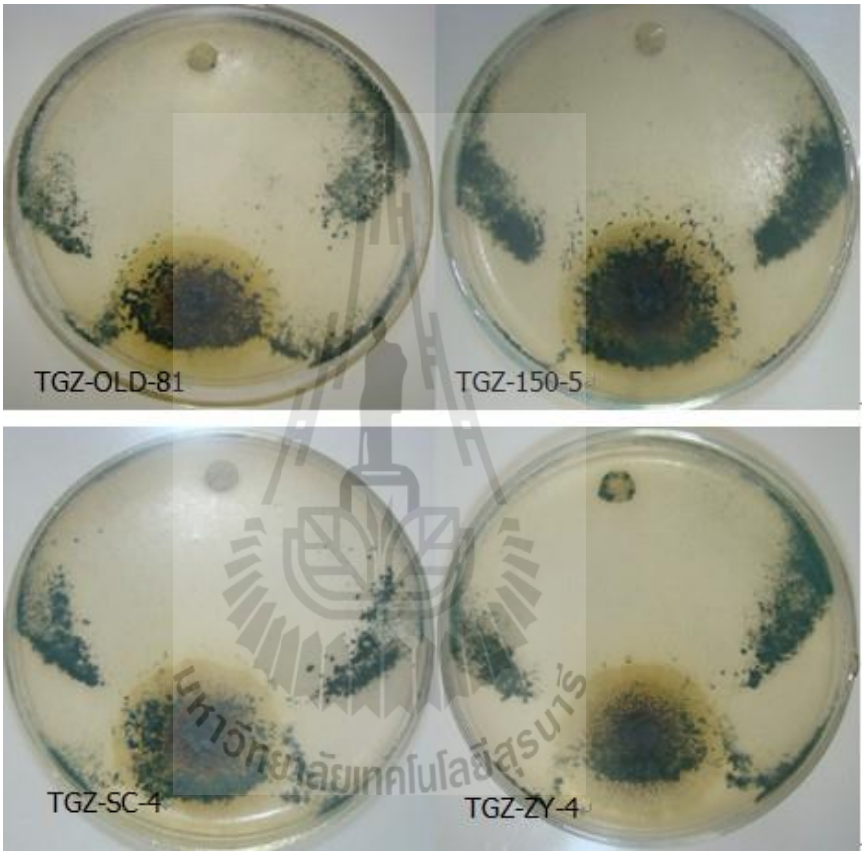

Figure 4.3 Inhibition of *Trichoderma* isolates on *Verticillium dahliae* mycelia after 4 days

Table 4.7 Inhibition of mycelial growth of *Verticillium dahliae* VGZ-HZ-4 and VGZ-XW-1 by 33*Trichoderma* isolates under a dual culture test condition

***Trichoderma* isolate**	**Mycelial inhibition[1] (%)**		**Average (%)**
	VGZ-HZ-4	**VGZ-XW-1**	
TGZ-SC-4	38.98a	37.76abd	38.37a
TGZ-150-5	36.51abc	40.20a	38.36a
TGZ-150-33	35.26abcd	38.98ab	37.12ab
TGZ-OLD-81	37.76ab	35.26bcd	36.51abc
TGZ-150-51	35.26abcd	37.76abc	36.51abc
TGZ-ZY-4	36.51abc	35.26bcd	35.89abcd
TGZ-150-38	33.98bcde	37.76abc	35.87abcd
TGZ-150-55	32.69cde	38.98ab	35.84abcd
TGZ-150-37	36.51abc	33.98cde	35.24bcd
TGZ-NKY-5	35.26abcd	33.98cde	34.62bcd
TGZ-150-41	33.98bcde	35.26bcd	34.62bcd
TGZ-150-4	35.23abcde	33.98cde	34.60bcd
TGZ-NKY-2	36.51abc	32.69def	34.60bcd
TGZ-150-1	33.98bcde	33.98cde	33.98cde
TGZ-150-6	35.26abcd	32.69def	33.98cde
TGZ-HZ-3	32.69cde	35.26bcd	33.98cde
TGZ-150-50	32.69cde	33.98cde	33.33def
TGZ-CH-3	31.35def	35.26bcd	33.30def
TGZ-CH-2	31.35def	31.35defg	31.35efg
TGZ-CH-4	32.69cde	27.05hi	29.87gh
TGZ-SC-5	28.58fg	30.00efgh	29.29ghi
TGZ-CH-1	28.58fg	30.00efgh	29.29ghi
TGZ-TC-3	25.63ghi	32.69def	29.16ghi
TGZ-SC-3	27.16gh	30.00efgh	28.58hij
TGZ-NKY-1	27.16gh	30.00efgh	28.58hij
TGZ-TC-4	27.05gh	30.00efgh	28.52hij
TGZ-NKY-7	24.10hij	32.69def	28.39hij
TGZ-TV-1	22.40ij	31.35defg	26.87ijk
TGZ-ZY-2	25.63ghi	27.16hi	26.39jk
TGZ-TV-2	22.40ij	28.58ghi	25.49k
TGZ-TV-3	20.44j	30.00efgh	25.22k
TGZ-NKY-8	24.10hij	25.63i	24.86k
TGZ-NKY-3	22.40ij	27.16hi	24.78k
Average	30.91	32.93	31.92
F-test	**	**	**
CV (%)	8.29	6.68	5.65

[1] Means in the same column followed by different letters are statistically different at P≤0.05 by DMRT. The inhibition percentage was calculated at 3days after the incubation.

4.4 Microsclerotia disintegration test

After pretesting the disinfection time for *Trichoderma* and *V. dahliae* , it was found that immersing paper dices containing the fungal propagules in 75% EtOH for 3 min followed by NaOCl for 5 min could kill the *Trichoderma* completely but not the microsclerotia of *V. dahliae*. This disinfection condition was subsequently applied for the microsclerotia disintegration test. The ten *Trichoderma* isolates having high mycelial inhibition ability could disintegrate microcsclerotia of both *V. dahliae* isolates completely (Table 4.8).

Table 4.8 Microsclerotia germination (MG) and disintegration (MD) of *Verticillium dahliae* by isolates of *Trichoderma*

Treatment	VGZ-HZ-4		VGZ-XW-1	
	MG	MD	MG	MD
TGZ-150-51	0	100	0	100
TGZ-OLD-81	0	100	0	100
TGZ-150-5	0	100	0	100
TGZ-150-37	0	100	0	100
TGZ-ZY-4	0	100	0	100
TGZ-NKY-2	0	100	0	100
TGZ-NKY-5	0	100	0	100
TGZ-150-33	0	100	0	100
TGZ-SC-4	0	100	0	100
TGZ-150-6	0	100	0	100
Control[1]	100	0	100	0

[1] Paper discs containing *V. dahliae* microsclerotia immersed in 75% EtOH for 3 min followed by 1% NaOCl for 5 min.

4.5 Small pot screening for *Trichoderma* efficacy

The results are as shown in Table 4.9. Most of the *Trichoderma* isolates could control the disease completely except the isolates TGZ-CH-4, TGZ-NKY-8, TGZ-TC-4 and TGZ-ZY-2.

Table 4.9 The efficacy of *Trichoderma* isolates in controlling wilt of potato cultivar Favorita inoculated with *Verticillium dahliae* VGZ-HZ-4 isolate in small pot test

Treatment	Wilt Incidence[3] (%)	Control Efficacy[3] (%)
TGZ-150-1	0c	100a
TGZ-150-4	0c	100a
TGZ-150-5	0c	100a
TGZ-150-6	0c	100a
TGZ-150-33	0c	100a
TGZ-150-37	0c	100a
TGZ-150-38	0c	100a
TGZ-150-41	0c	100a
TGZ-150-50	0c	100a
TGZ-150-51	0c	100a
TGZ-150-55	0c	100a
TGZ-CH-2	0c	100a
TGZ-CH-1	0c	100a
TGZ-CH-3	0c	100a
TGZ-CH-4	50b	50b
TGZ-HZ-3	0c	100a
TGZ-NKY-1	0c	100a
TGZ-NKY-2	0c	100a
TGZ-NKY-3	0c	100a
TGZ-NKY-5	0c	100a
TGZ-NKY-7	0c	100a
TGZ-NKY-8	25bc	75ab
TGZ-OLD-81	0c	100a
TGZ-SC-3	0c	100a
TGZ-SC-4	0c	100a
TGZ-SC-5	0c	100a
TGZ-TC-3	0c	100a
TGZ-TC-4	25bc	75ab
TGZ-TV-1	50b	50b
TGZ-TV-2	25bc	75ab
TGZ-TV-3	0c	100a

Table 4.9 (Continued

Treatment	Wilt Incidence[3] (%)	Control Efficacy[3] (%)
TGZ-ZY-2	50b	50b
TGZ-ZY-4	0c	100a
Control A[1]	100a	0c
Control B[2]	0c	100a
F-test	**	**
CV (%)	22.18	22.18

[1] Control A: *Verticillium* and without *Trichoderma*

[2] Control B: Without *Verticillium* and *Trichoderma*

[3] Means in the same column followed by different letters are statistically different at P≤0.05 by DMRT.

4.6 Efficacy of *Trichoderma* to control *Verticillium* wilt in the greenhouse

From Table 4.10 it can be seen that the number of survived plants were not different if the *Trichoderma* were applied before the *V. dahliae* inoculation. Most of the plants had 100% survival at all *Trichoderma* dosages used in the test. But when they were applied after the *V. dahliae* inoculation TGZ-150-5 and TGZ-ZY-4 appeared to be most effective at the dosage as low as 20 g.kg soil $^{-1}$ giving 40% of the survived plants. Increasing dosages of these 2 isolates seem to decrease number of survived plants compared to that of the 20 g.kg soil $^{-1}$ dosage

Table 4.10 Survived percentage of Favorita potato plants inoculated with *Verticillium dahliae* VGZ-HZ-4 and treated with different dosages (g.kg soil^{-1}) of 4 *Trichoderma* isolates under a greenhouse condition

Treatment[1]	Survived plant (%)[2]							
	Pre *V. dahliae* infection				Post *V. dahliae* infection			
	10	**20**	**40**	**80**	**10**	**20**	**40**	**80**
V.&TGZ-SC-4	93.75	100	100	100	6.25b	20c	5c	19.58b
V.&TGZ-OLD-81	100	100	100	100	11.25b	20bc	17.5c	32.08b
V.&TGZ-150-5	100	100	100	100	5b	40b	30.83b	24.58b
V.&TGZ-ZY-4	100	100	100	100	5b	40b	30.83b	24.58b
No.V.&TGZ-SC-4	100	100	100	100	100a	100a	100a	100a
No V.&TGZ-OLD-81	100	100	100	100	100a	100a	100a	100a
No V.&TGZ-150-5	100	100	100	100	100a	100a	100a	100a
No V.&TGZ-ZY-4	100	100	100	100	100a	100a	100a	100a
F-test	ns	ns	ns	ns	**	**	**	**
CV(%)	4.45				12.97	16.78	16.15	17.73

[1] V. = *Verticillium dahliae* VGZ-HZ-4

[2] Means in the same column followed by different letters are statistically different at P≤0.05 by DMRT. NS = nonsignificant

CHAPTER V

CONCLUSION AND DISCUSSION

Results of the experiments to screen for effective *Trichoderma* isolates for controlling *Verticillium* wilt in potato can be concluded as follows:

5.1 Twenty isolates of *Verticillium dahliae* could be isolated from diseased samples from various potato growing areas in Guizhou. The isolates were similar in morphology and growth characteristics but different in pathogenicity reflecting their diversity. Among them, isolate VGZ-HZ-4 gave the highest wilt incidence of 39.71% while VGZ-HZ-1 gave the lowest incidence of 9.91% when averaged from the 2 potato cultivars inoculated with the 2 methods, root dipping and microsclerotia inoculation. It is interesting that both of them came from the same location, Hezhang but VGZ-HZ-1 was isolated from diseased WeiYu-3 potato cultivar. Biodiversity among *V. dahliae* isolates is a common phenomena that has been observed by many researchers (Schubert *et al.*, 2008; Steven *el al.*, 2009). Both crop species and planting areas could have an effect on the diversity (Steven *et al.*, 2009). Such finding indicate the necessity of screening *V. dahliae* for pathogenicity before any research of this nature could be conducted.

5.2 For the inoculation, when the results were combined analysed, microsclerotia inoculation appeared to have a higher efficacy than that of the root dipping using conidia suspension. However, when the single factor was analysed it was obvious that both inoculation methods were equally effective on the Favorita

cultivar and only on Hui-2 that root dipping was less effective. It appears that inoculation methods do not affect the efficacy if the test cultivars are susceptible but will have a significant effect if the test cultivars are resistant. Results of this observation could be used to explain why some researchers were more successful using root dip inoculation (Gordon, Shaw and Larson, 2005) while other found that microsclerotia inoculation was better (Maas *et al.*, 1985). Both inoculation methods have advantages and disadvantages. The microsclerotia inoculation is more natural considering that microsclerotia are the fungal propagules over seasoning in the soil and are the main inocula that start the disease cycle (Isaac, 1946; Bejarano-Alcázar, Termorshuizen and Jiménez-Díaz, 1999). But to prepare microsclerotia as inocula is rather difficult and time consuming. In contrast to the root dip method in which conidia suspension is used as inoculum, the conidia can be prepared at ease but they may not survive the field condition. Based on the result of this experiment, root or seed dipping in conidia suspension was used in the upcoming experiment because Favorita would be used as test cultivar.

5.3 Between the 2 cultivars tested, it was evident that Favorita was highly susceptible while Hui-2 was highly resistant to *V. dahliae*. The response observes in the experiment had confirmed what had been observed in the field. Favorita, although the most popular and widely grown in Guizhou always had bad records with *Verticillium* wilt. It was also reported to be susceptible to *Phytophthora imfestans* (Li and Wang, 2006). From results of this experiment growing Favorita should be discouraged in Guizhou and be replaced by Hui-2. It is interesting to note that Hui-2 had 100% survival when inoculated with conidia suspension of many *V. dahliae* isolates when those isolates caused 100% wilt incidence in Favorita inoculated in the

same way. The wilt incidence found on Hui-2 mainly came from the result of microsclerotia inoculation. There should be further investigation to find out why Hui-2 was susceptible when inoculated with microsclerotia but resistant to infection by conidia.

5.4 The 21 *Trichoderma* isolates obtained from the soil samples and 11 single-spore isolates obtained from re-isolation of the TGZ-150 isolate preserved at GZIPP, could inhibit mycelial growth of both VGZ-HZ-4 and VGZ-XW-1 *V. dahliae* isolates but with different degree of efficacy. Most of the single-spore isolates and the TGZ-OLD-81 isolate had higher than the average mycelial inhibition percentage of all test isolates. Among them, only TGZ-SC-4 isolates from Shuicheng soil had a better efficacy than that of the GZIPP isolates. This isolate, TGZ-OLD-81 and most single-spore isolates could overgrow the *V. dahliae* within 2 days at 28^{o}C. The selected 10 isolates of this group could 100% disintegrate the *V. dahliae* microsclerotia. Both mycelia inhibition and microsclerotia disintegration are important mode of actions in controlling fungal disease (Hayfa *et al.*, 2009; El-Rafai *et al.*, 2003) apart from other characters such as mycoparasitism (Punja and Utkehede, 2003; Thornton *et al.*, 2002) and antibiosis (Menendez and Godeas, 1998; Ghisalberti, 1991). Considering microsclerotia an important source of initial inoculum, the ability of *Trichoderma* isolates to disintegrate them should be most desirable as a biocontrol agent.

5.5 After being tested under a small pot condition, most of the 33 isolates could control the wilt disease at 100% efficacy except TGZ-CH-4, TGZ-NKY-8, TGZ-TC-4, TGZ-TV-1, TGZ-TV-2 and TGZ-ZY-2 that gave less than 100% efficacy. These were isolates obtained from soil samples. The 10 isolates capable of disintegrating the miscrosclerotia also had 100% efficacy in controlling the disease.

5.6 After passing through all preliminary screening, the top 4 *Trichoderma* isolates were tested under a greenhouse condition using VGZ-HZ-4 as the representative *V. dahliae* isolate, Favorita as the test potato cultivar and seed dipping in conidia suspension as the inoculation method. When *Trichoderma* was applied to the soil at sowing time prior to *V. dahliae* inoculation, all 4 *Trichoderma* isolates could control the disease at 100% efficacy regardless of the *Trichoderma* dosages. But when they were applied 20 days after the seed *V. dahliae* inoculation, TGZ-150-5 and TGZ-ZY-4 performed better than TGZ-OLD-81 and TGZ-SC-4 but gave the maximum controlling efficacy of only 40% at 20 g.kg soil-1. Increasing dosages of these 2 isolates appeared to decrease number of the survived plants. To attain the same level of controlling efficacy in TGZ-OLD-81, the dosage of up to 80 g.kg soil-1 had to be applied. Results of this experiment has indicated clearly that application time of *Trichoderma* is most essential for the controlling measure effectiveness. Most of the successful reported cases were when *Trichoderma* was applied before the pathogen infection (Marois *et al.*, 1982; Kexiang *et al.*, 2002; Chen *et al.*, 2011). *T. harzianum* was used as seed dressing or applied into sowing row (Ordentlich, Nachmias and Chet, 1990) to control *Verticillium* wilt in potato. The success of this method could come from the ability of *Trichoderma* to colonize the rhizosphere which is the initial size for *Verticillium* colonization before the infection (Huisman, 1988). Since *Trichoderma* can not enter the roots, it can not exert its antagonistic activity to the pathogen that has already invaded the roots resulting in the poor controlling efficacy observed when *Trichoderma* was applied post infection. The observed 40% controlling efficacy in this experiment could have come from the induced resistance capacity of isolates TGZ-150-5 and TGZ-ZY-4. Such capacity in *Trichoderma* is one

of its mode of action in controlling plant disease (Woo *et al.*, 2005; Khan *et al.*, 2004; Malmierca *et al.*, 2012; Elad, 2000). It is interesting to note that the isolate TGZ-SC-4 which showed the highest *V. dahliae* mycelial inhibition activity performed less well in the greenhouse test and ranked the poorest among the 4 *Trichoderma* isolates. This might due to it is a poorer plant resistance inducer because when it was applied prior to *V. dahliae* infection, it performed as well as the other isolates. The good performance observed in TGZ-ZY-4, a newly isolated strain indicated that it could be developed into a commercial isolate. The consistent good efficacy observed in TGZ-150-5 and TGZ-OLD-81 has confirmed the good performance of the GZIPP isolates. Both of these isolates are *T. harzianum* that have been tested and used for controlling soil-borne diseases since 2001 (Yan *et al.*, 2001). Their efficacy has been well recognized in many cases (Wu, Yan and Lu, 2002; Chen *et al.*, 2011), reflecting their stability as a biological control agent.

5.7 It could be concluded at this point that the most effective *Trichoderma* isolates for controlling *Verticillium* wilt in potato are TGZ-150-5 and TGZ-ZY-4. To gain their maximum efficacy they should be applied before the *V. dahliae* infection at the dosage of 20 g.kg soil^{-1}. There should be further investigation on field application of these 2 strains before they could be recommended for commercial use.

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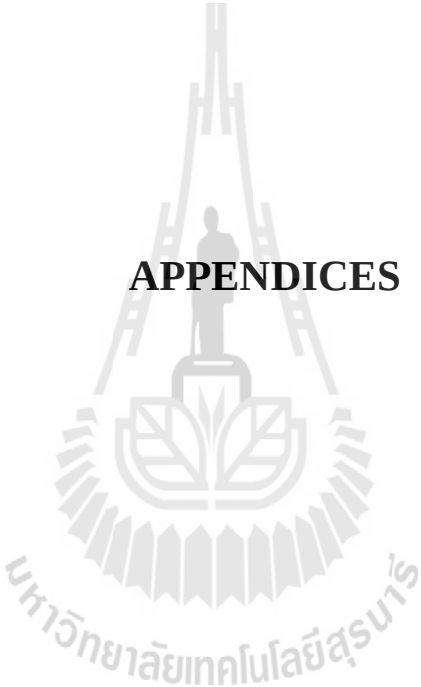
APPENDICES



Appendix figure 1: Soil sterilization (left) and high pressure sterilizer (right)

Appendix figure 2: Small pot screening

Appendix figure 3: Character of *Trichoderma* isolates in the wheat medium

Appendix figure 4: Potao seedling and root dipping inoculation

Appendix figure 5: Pot test in greenhouse conditional

BIOGRAPHY

Xiaojun Chen was born on June 29, 1979 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 Institute of Plant Protection, which is member of Guizhou Academy of Agricultural Sciences. In 2008, 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.