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In vitro inhibition of pathogenic *Verticillium dahliae*, causal agent of potato wilt disease in China by *Trichoderma* isolates

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Twenty (20) of *Verticillium dahliae* were isolated from wilted potato specimens collected from six districts in Guizhou, China. All the isolates were evaluated for pathogenicity on two potato cultivars, Favorita (susceptible) and Hui-2 (resistant) using the root dip inoculation (RDI) and microsclerotia inoculation (MI). All of the *V. dahliae* isolates appeared to be pathogenic on both cultivars but VGZ-HZ-4 isolate gave the highest wilt incidence comparing to the others, seconded by VGZ-SC-1 and VGZ-XW-1. Combined analysis of wilt incidence resulting from using two inoculation methods for VGZ-HZ-4 and VGZ-XW-1 isolates on the two potato cultivars showed that the MI gave a higher wilt incidence than that of the RDI and cultivar Favorita had a higher wilt incidence than that of Hui-2. These two *V. dahliae* isolates were further used as representative isolates for mycelial inhibition (MyI) test with 33 *Trichoderma* isolates under a dual culture condition on potato dextrose agar plate. The 33 *Trichoderma* isolates consisting of 21 isolates isolated from potato soils from seven districts of Guizhou, 11 isolates from single spore isolates of the TGZ-150 isolate preserved at Guizhou Institute of Plant Protection (GZIPP) and one isolate TGZ-OLD-81 also preserved at the GZIPP. Most of the single spore isolates and TGZ-SC-4 were found to have higher MyI efficiency than that of the rest. The results indicate that the *Trichoderma* isolates in this study have initial modes of action of biological control to protect potato crop against *V. dahlia*.

Key words: *Trichoderma*, potato wilt disease, growth inhibition, *Verticillium dahliae*, antagonistic fungi.

INTRODUCTION

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), asympto-

matic 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 possesses 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 (Brunner et al., 2005).

Potato (*Solanum tuberosum* L.) is the World's and China's fourth largest staple crop after rice, wheat and maize. 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). 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). *Verticillium* wilt is a complex disease caused by many species of *Verticillium*, such as *Verticillium dahliae* Kleb or *Verticillium alboatrum* (Francl et al., 1990; 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 (*S. 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 et al., 2004; Uppal et al., 2008; Cirulli, 1981). 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 USA (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 et al., 2008) and can survive in the soil for 5 to 14 years (http://www.crop.cri.nz/home/ products-services/publications/ broadsheets/126-Potato.pdf). The management of *Verticillium* wilt is challenging not only due 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 (Hanson, 2000; Kexiang et al., 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 (Gamliel et al., 2000; Zamanian et al., 2005). *Trichoderma* spp. are widely used as commercial biofungicides for control of soil-borne and foliar pathogens (Chet and Baker, 1981; Cook and Baker, 1982; Papavizas, 1985; Verma et al., 2007; Buensanteai et al., 2010; Akinbode and Ikotun, 2011). In addition, plant pathogens directly affected through antibiosis and mycoparasitism, *Trichoderma* spp. can colonize roots and trigger systemic resistance against bacterial and fungal pathogens (Liansheng and Weihua, 2000; Harman et al., 2004). Induced resistance caused by treating plants with a biologically active elicitor is the phenol-menon of priming and sensitizing plants to exhibit a more rapid and elevated expression of defense-related responses upon pathogen infection compared to unprimed plants. These responses may include an accumulation of PR proteins associated with the SA-dependent and the pathogen-induced JA-dependent pathways, as well as phenylalanine ammonia-lyase and redox regulating proteins (Mittler et al., 2004; Fobert and Despres, 2005; Heil and Silva Bueno, 2007; Buensanteai et al., 2010).

The aim of this study was to evaluate the efficacy of *Trichoderma* strains for antagonistic activity on the fungal pathogen *V. dahliae,* causal agent of potato wilt disease.

MATERIALS AND METHODS

Pathogen isolation

Ten (10) 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 to 45 s and then dipped in 70% ethyl alcohol for 20 to

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30 s. 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. The stem sections were incubated for 14 to 20 days at 22 to 24°C as reported by Slattery and Eide (1980). After four days, the *V. dahliae* hyphal tip grown out from each piece of tissue of potato was picked and transferred onto the new potato dextrose agar (PDA) for further experiment.

Pathogenicity test of *V. dahliae* isolates

Root dip inoculation (RDI)

Twenty (20) of the *V. dahliae* isolates obtained were tested for their pathogenicity in potato seedlings. All cultures were grown on PDA at 24±2°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 haemacytometer, the inoculum concentration was adjusted to 10^7 conidia/ml. Potato seedlings of the Favorita and Hui-2 cultivars 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 submerged for 60 min in conidia suspension. The inoculated seedlings were subsequently transplanted into 20 cm pots with sterilized soil (autoclaved 1 h, 121°C -or whatever it is). Three replications of five plants for each isolate were used. The plants were kept on a room bench at 21 to 23°C. Daylight was supplemented by fluorescent lamps to provide a 12 h day length; wilt incidence was checked at four weeks after inoculation.

Microsclerotia inoculation (MI)

The same 20 isolates of *V. dahliae* were used for this experiment. After microsclerotia having formed on PDA, the whole agar piece was removed from the plate and put in the soil beneath the roots of transplanted potato seedlings of the Favorita and Hui-2 cultivars in a 20 cm pot. Three replications of five plants for each isolate were used. The plants were kept under the same condition as in previous experiment; wilt incidence was observed four weeks after inoculation. The experimental trials were conducted following a three-factor complete randomized design (CRD).

Trichoderma origin and isolation

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 to 50°C) (Williams et al., 2003).

***Trichoderma* isolation from soil**

Ten (10) soil samples of each field 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 soil 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, two 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.

Single spore isolates

Trichoderma harzianum TGZ-150 was isolated from rhizosphere soil of tobacco damaged by tobacco root rot (*Fusarium oxysporum*) in Bijie city of Guizhou province in 2011 and preserved at the Plant Pathology Laboratory of Guizhou Institute of Plant Protection (GZIPP). The 0.03 ml of TGZ-150 *Trichoderma* spore suspension (10^3 conidia/ml) was spread on 10 PDA medium, incubated at 28±2°C for 12 h and examined frequently every 12 h under a microscope for germinating spores which were picked and transferred with a sterile cork borer to fresh PDA. The single spore isolates were maintained in PDA slant for the future experiment.

In vitro* inhibition of *V. dahliae* by *Trichoderma

Mycelial inhibition (Myl)

Trichoderma isolates used for this study were from two origins. One was the 11 single spore isolates of TGZ150, and the strain TGZ-OLD-81 preserved at GZIPP, the others were those isolated from the fields. The *T. harzianum* and the *V. dahliae* isolates tested were VGZ HZ4 and VGZ XW1 which previously showed the highest levels of pathogenicity in potato. *Trichoderma* isolates were tested *in vitro* for their highest antagonistic ability over *V. dahliae* colony in dual culture. One mycelial disc (5 mm) of each *Trichoderma* isolate and *V. dahliae* was put together in a Petri dish with PDA medium, 6 cm apart. Three replications (dishes) were used for each *Verticillium*-*Trichoderma* combination. The *V. dahliae* mycelial growth inhibition rate was calculated three days after incubation. The percent of growth inhibition was calculated as follows:

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

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

Microsclerotia disintegration (MD)

Based on the fact that 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 (5 mm) was put at the edge of each growing colony of V. dahliae in order to collect microsclerotia. Numerous microsclerotia were produced on the

Table 1. Isolates of *Verticillium dahliae* isolated from the wilted potato grown in Guizhou Province, China used in the experiment.

Isolate	Site	Host	Isolate	Site	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.

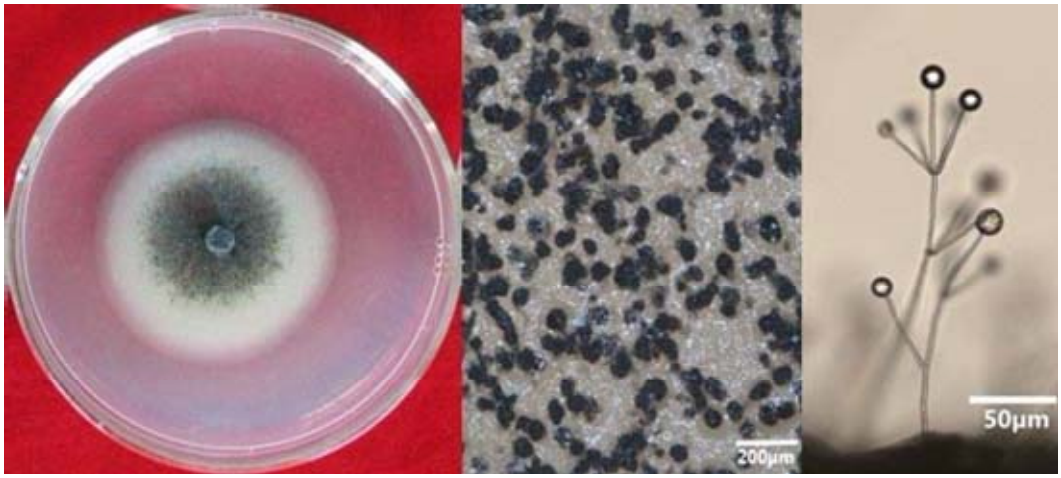

Figure 1. Colony character (left), microsclerotia (middle) and conidia (right) of *Verticillium dahliae* on APDA medium.

paper disc after one week. Ten (10) 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. After 1 day, 30 MPDs were put at the edge of the growing colony in three 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 after disinfectant (75% ethyl alcohol 3 min and 1% NaOCl 5 min) 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.

Statistics analysis

The data was analyzed by SPSS 16.0 software. To ensure that 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. Statistical differences were determined by DMRT (Duncan) test.

RESULTS

Isolation of *V. dahliae* and *Trichoderma*

Twenty (20) isolates of *V. dahliae* were obtained from the planting areas of Guizhou province (Table 1). Colony character showed white mycelia and produced black microsclerotia on APDA (Figure 1). From the soil samples collected from different location in Guizhou, 21 *Trichoderma* isolates were obtained (Table 2). Additional 11 isolates were single-spore isolated from *T. harzianum* TGZ-150, a commercial isolate preserved at GZIPP. All

Table 2. Isolates of *Trichoderma* from the GZIPP collection and obtained from the soil in Guizhou Province, China.

Isolate	Site	Isolate	Site
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	
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	Single spore isolate of TGZ-150[2]
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.

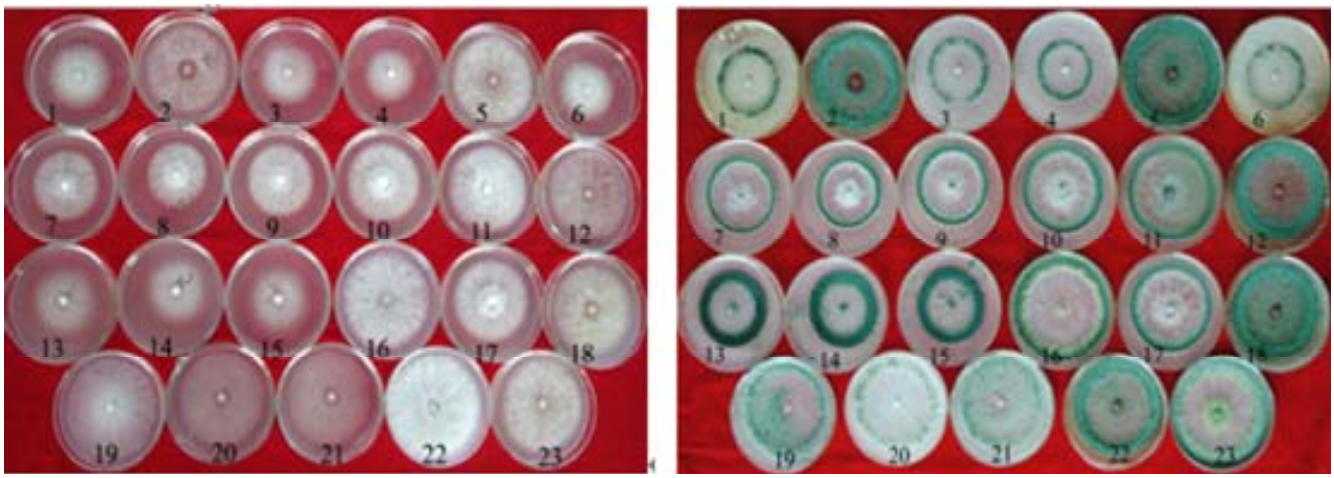

Figure 2. The character of *Trichoderma* isolates cultured for 2 days (left) and 7 days (right) on PDA. (1 TGZ-TV-1, 2 TGZ-150-37, 3 TGZ-TV-2, 4 TGZ-TV-3, 5 TGZ-150-5, 6 TGZ-150-33, 7 TGZ-CH-1, 8 TGZ-CH-2, 9 TGZ-CH-3, 10 TGZ-CH-4, 11 TGZ-ZY-2, 12 TGZ-ZY-4, 13 TGZ-NKY-1, 14 TGZ-NKY-2, 15 TGZ-NKY-3, 16 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).

these isolates and TGZ-OLD-81 were included in the up-coming experiment (Figure 2).

Pathogenicity test of *V. dahliae* isolates

The 20 isolates of *V. dahliae* were tested for pathogenicity on two potato cultivars by root dip inoculation (RDI) and microsclerotia inoculation (MI). All of the *V. dahliae* isolates could infect potato cultivar Favorita (Table 3). When results of pathogenicity of the 20 *V. dahliae* isolates on the two potato cultivars 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

Table 3. Wilt incidence on 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	26.67^{ab}	33.33^{bcd}	0.00^{a}	6.67^{ab}
VGZ-SC-2	40.00^{ab}	40.00^{abcd}	6.67^{a}	6.67^{ab}
VGZ-SC-3	46.67^{ab}	33.33^{bcd}	0.00^{a}	0.00^{b}
VGZ-SC-4	33.33^{ab}	40.00^{abcd}	13.33^{a}	13.33^{ab}
VGZ-SC-5	46.67^{ab}	46.67^{abcd}	0.00^{a}	6.67^{ab}
VGZ-SC-6	20.00^{bc}	46.67^{abcd}	6.67^{a}	6.67^{ab}
VGZ-SC-7	46.67^{ab}	53.33^{abc}	0.00^{a}	0.00^{b}
VGZ-HZ-1	20.00^{bc}	6.67^{ef}	0.00^{a}	6.67^{ab}
VGZ-HZ-3	53.33^{ab}	46.67^{abcd}	6.67^{a}	0.00^{b}
VGZ-HZ-4	60.00^{a}	73.33^{a}	20.00^{a}	20.00^{a}
VGZ-HZ-9	20.00^{bc}	20.00^{de}	0.00^{a}	0.00^{b}
VGZ-HZ-10	40.00^{ab}	66.67^{ab}	6.67^{a}	0.00^{b}
VGZ-CS-1	66.67^{a}	73.33^{a}	13.33^{a}	13.33^{ab}
VGZ-CS-2	40.00^{ab}	53.33^{abc}	0.00^{a}	6.67^{ab}
VGZ-CS-5	33.33^{ab}	26.67^{cd}	0.00^{a}	13.33^{ab}
VGZ-WN-1	40.00^{ab}	53.33^{abc}	13.33^{a}	13.33^{ab}
VGZ_WN-2	40.00^{ab}	60.00^{abc}	6.67^{a}	6.67^{ab}
VGZ-NKY-2	46.67^{ab}	46.67^{abcd}	6.67^{a}	13.33^{ab}
VGZ-NKY-4	20.00^{bc}	40.00^{abcd}	0.00^{a}	0.00^{b}
VGZ-XW-1	46.67^{ab}	60.00^{abc}	13.33^{a}	20.00^{a}
Control[2]	0.00^{c}	6.67^{f}	0.00^{a}	0.00^{b}
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.*

which gave wilt incidence that were not statistically different (37.41 and 34.62%, respectively) (Table 4). The combined average wilt incidence of RDI and MI on the cultivar Favorita was higher than cultivar Hui-2 (Table 5) which was 39.63 and 8.62%, respectively. Table 6 shows the combined wilt incidence resulting from the use of 2 inoculation techniques on the two cultivars. It appears that the MI gave a higher wilt incidence (25.92%) compared to that of the RDI. VGZ-HZ-4 and VGZ-XW-1 were selected as representative isolates for further experiment.

In vitro inhibition of *V. dahliae* by *Trichoderma* isolates

In this experiment, the 33 *Trichoderma* isolates were tested for mycelia growth inhibition of the *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 four days (Figure 3). Most of the *Trichoderma* isolates grew so fast and had the averaged colony radius of 41.86, 54.08 and 65.98 mm after two, three and four days, respectively. After seven days, all of them could produce spores at the average of 1.38×10^{10} cfu/dish.

After three days of dual culture at 28°C, most of the isolates had performed noticeably well on inhibition ability. The inhibition percentage measured at three days after the dual culture is shown in Table 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%.

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*

Table 4. Combined wilt incidence on favorita and Hui-2 potato cultivars inoculated by root dip technique and microsclerotia inoculation with different *Verticillium dahliae* isolates.

V. dahlia isolate	Wilt incidence (%)[1]
VGZ-HZ-4	39.71[a]
VGZ-CS-1	37.41[ab]
VGZ-XW-1	34.62[ab]
VGZ-WN-1	30.30[abcd]
VGZ-NKY-2	28.18[abcd]
VGZ-SC-4	27.42[abcd]
VGZ-WN-2	26.93[bcde]
VGZ-HZ-10	25.77[cde]
VGZ-HZ-3	24.81[cde]
VGZ-SC-2	23.95[cde]
VGZ-CS-2	23.75[cde]
VGZ-SC-5	23.66[cde]
VGZ-SC-7	22.50[de]
VGZ-SC-6	20.68[def]
VGZ-SC-3	19.52[def]
VGZ-SC-1	18.66[def]
VGZ-CS-5	18.47[def]
VGZ-NKY-4	15.29[ef]
VGZ-HZ-9	10.97[fg]
VGZ-HZ-1	9.91f[g]
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 5. Combined wilt incidence on two potato cultivars inoculated with *Verticillium dahliae* by two inoculation methods.

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

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

completely but not the microsclerotia of *V. dahliae*. This disinfection condition was subsequently applied for the microsclerotia disintegration test. The 10 *Trichoderma* isolates having high mycelial inhibition ability could disintegrate microcsclerotia of both *V. dahliae* isolates completely (Table 8).

DISCUSSION

Twenty (20) isolates of *V. 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 the averaged from the 2 potato cultivars were inoculated with the two methods, root dipping (RDI) and microsclerotia inoculations (MI). 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). Both crop species and planting areas could have an effect on the diversity (Steven et al., 2009). Such finding indicates the necessity of screening *V. dahliae* for pathogenicity before any research of this nature could be conducted.

Table 6. Combined wilt incidence of two inoculation methods on two potato cultivars.

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

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

For the inoculation, when the results were combined and analyzed, MI appeared to have a higher efficacy than that of the RDI using conidia suspension. However, when the single factor was analyzed it was obvious that both inoculation methods were equally effective on the Favorita cultivar and only on Hui-2 that RDI was less effective. It appears that inoculation methods do not affect the efficacy if the test cultivar is susceptible but will have a significant effect if the test cultivar is resistant. Results of this observation could be used to explain why some researchers were more successful using RDI while other found that MI was better (Maas et al., 1985). Both inoculation methods have advantages and disadvantages. The MI 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 (Maas et al., 1985). But to prepare microsclerotia as inocula is rather difficult and time consuming. In contrast to the RDI 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.

As regard to the two cultivars tested, it was evident that

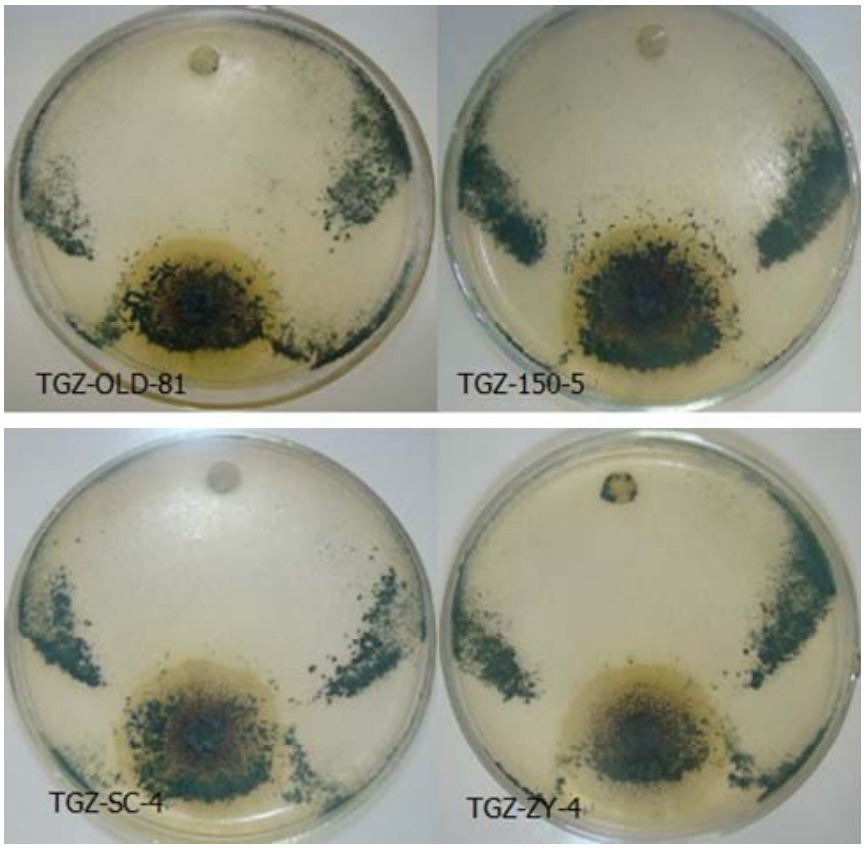

Figure 3. Inhibition of mycelia growth of *Verticillium dahliae* by *Trichoderma* isolates after 4 days.

Table 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	38.98^{a}	37.76^{abd}	38.37^{a}
TGZ-150-5	36.51^{abc}	40.20^{a}	38.36^{a}
TGZ-150-33	35.26^{abcd}	38.98^{ab}	37.12^{ab}
TGZ-OLD-81	37.76^{ab}	35.26^{bcd}	36.51^{abc}
TGZ-150-51	35.26^{abcd}	37.76^{abc}	36.51^{abc}
TGZ-ZY-4	36.51^{abc}	35.26^{bcd}	35.89^{abcd}
TGZ-150-38	33.98^{bcde}	37.76^{abc}	35.87^{abcd}
TGZ-150-55	32.69^{cde}	38.98^{ab}	35.84^{abcd}
TGZ-150-37	36.51^{abc}	33.98^{cde}	35.24^{bcd}
TGZ-NKY-5	35.26^{abcd}	33.98^{cde}	34.62^{bcd}
TGZ-150-41	33.98^{bcde}	35.26^{bcd}	34.62^{bcd}
TGZ-150-4	35.23^{abcde}	33.98^{cde}	34.60^{bcd}
TGZ-NKY-2	36.51^{abc}	32.69^{def}	34.60^{bcd}
TGZ-150-1	33.98^{bcde}	33.98^{cde}	33.98^{cde}
TGZ-150-6	35.26^{abcd}	32.69^{def}	33.98^{cde}
TGZ-HZ-3	32.69^{cde}	35.26^{bcd}	33.98^{cde}
TGZ-150-50	32.69^{cde}	33.98^{cde}	33.33^{def}

Table 7. Contd.

TGZ-CH-3	31.35^{def}	35.26^{bcd}	33.30^{def}
TGZ-CH-2	31.35^{def}	31.35^{defg}	31.35^{efg}
TGZ-CH-4	32.69^{cde}	27.05^{hi}	29.87^{gh}
TGZ-SC-5	28.58^{fg}	30.00^{efgh}	29.29^{ghi}
TGZ-CH-1	28.58^{fg}	30.00^{efgh}	29.29^{ghi}
TGZ-TC-3	25.63^{ghi}	32.69^{def}	29.16^{ghi}
TGZ-SC-3	27.16^{gh}	30.00^{efgh}	28.58^{hij}
TGZ-NKY-1	27.16^{gh}	30.00^{efgh}	28.58^{hij}
TGZ-TC-4	27.05^{gh}	30.00^{efgh}	28.52^{hij}
TGZ-NKY-7	24.10^{hij}	32.69^{def}	28.39^{hij}
TGZ-TV-1	22.40^{ij}	31.35^{defg}	26.87^{ijk}
TGZ-ZY-2	25.63^{ghi}	27.16^{hi}	26.39^{jk}
TGZ-TV-2	22.40^{ij}	28.58^{ghi}	25.49^{k}
TGZ-TV-3	20.44^{j}	30.00^{efgh}	25.22^{k}
TGZ-NKY-8	24.10^{hij}	25.63^{i}	24.86^{k}
TGZ-NKY-3	22.40^{ij}	27.16^{hi}	24.78^{k}
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 3 days after the incubation.

Table 8. Microsclerotia germination (MG) and disintegration (MD) of *Verticillium dahliae* by isolates of *Trichoderma*.

***Trichoderma* isolate**	**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.

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.* 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 MI. There should be

further investigation to find out why Hui-2 was susceptible when inoculated with microsclerotia but resistant to infection by conidia.

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 two days at 28°C. The selected 10 isolates of this group could be 100% disintegrate the *V. dahliae* microsclerotia. The rapid growth of *Trichoderma* over *Verticillium* suggests that competition for space and nutrients could be one of the mechanisms implied in the antagonist action of Trichoderma in this essay. Both mycelia inhibition and microsclerotia disintegration are important mode of actions in controlling fungal disease (Hartman et al., 1981; Papavizas, 1985) apart from other mechanisms such as mycoparasitism (Papavizas, 1985; Harman et al., 2004; Mishra, 2010) and antibiosis (Papavizas, 1985). Considering microsclerotia an important source of initial inoculum, the ability of *Trichoderma* isolates to disintegrate them should be most desirable as a biocontrol agent.

Conclusion

The results of this experiment 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. There should be further investigation on field application of these two strains before they could be recommended for commercial use in the near future.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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