Appl. Ent. Phytopath.
Vol. 84, No. 2, March, 2017

Biocontrol of Verticillium wilt of potato caused by *Verticillium dahliae* using selected biocontrol agents

J. AMINI✉
Associate Professor, Department of Plant Protection, College of Agriculture, University of Kurdistan, P. O. Box 416, Sanandaj, Iran
(Received: May 2016; Accepted: January 2017)

Abstract

Verticillium wilt of potato is a major limiting factor in potato production. In this study, a total of 14 biocontrol agents, consisting of seven different species (*Trichoderma harzianum*, *Trichoderma deliquescens*, *Fusarium oxysporum*, *Talaromyces flavus*, *Bacillus subtilis*, *Pseudomonas fluorescens* and *Serratia marcescens*) were examined against *Verticillium dahliae* the causal agent of potato wilt *in vitro* and greenhouse conditions. Antagonistic effects were evaluated through volatile and non-volatile metabolite productions *in vitro*. All antagonists caused significant reduction in growth of *V. dahliae* compared to the control. The maximum antagonistic effect was observed in isolates of *T. flavus* with inhibition zones ranging from 65.4 to 66.7% by dual culture method. The ability of biocontrol agents varied in production of siderophore, protease, cyanide hydrogen and indole acetic acid. Results of the greenhouse study 75 days after sowing indicated that all biocontrol agents reduced disease severity and vascular discoloration of Verticillium wilt at different rates. Accordingly, *Trichoderma flavus* TFPV24 was the most effective in reducing disease severity and vascular discoloration of potato Verticillium wilt by 76% and increasing yield by 171% compared to the untreated control. In general, in both *in vitro* and greenhouse assay, isolates of *T. flavus* were the most effective antagonist and *Serratia marcescens* has the least antagonistic effect. The overall results of this study showed that isolates of *T. flavus* have high efficacy in controlling potato Verticillium wilt.

Key words: Biocontrol, *Fusarium oxysporum*, Verticillium wilt, *Talaromyces flavus.*

کنترل بیولوژیک بیماری پژمردگی ورتیسیلیومی سیب زمینی با استفاده از عوامل بیوکنترل انتخابی

جهانشیر امینی✉
دانشیار گروه گیاهپزشکی دانشکده کشاورزی، دانشگاه کردستان

چکیده

پژمردگی ورتیسیلیومی سیب زمینی از عوامل محدود کننده کشت و تولید سیب زمینی است. در این تحقیق ۱۴ عامل کنترل کننده زیستی از هفت گونه متفاوت شامل *Trichoderma harzianum*، *Trichoderma deliquescens*، *Fusarium oxysporum*، *Talaromyces flavus*، *Bacillus subtilis*، *Pseudomonas fluorescens* و *Serratia marcescens* علیه قارچ بیمارگر *Verticillium dahliae* در شرایط آزمایشگاه و گلخانه مورد آزمایش قرار گرفت. چگونگی تاثیر عوامل بیوکنترل علیه قارچ بیمارگر به روش‌های کشت متقابل، تولید مواد فرار و مایع خارج سلولی بررسی گردید. تمام عوامل بیوکنترل مذکور سبب کاهش قابل توجه رشد پرگنه قارچ بیمارگر در شرایط آزمایشگاه شدند. در آزمایش کشت متقابل جدایه‌های *T. flavus* بیشترین تاثیر بازدارندگی (۶۶/۷ – ۶۵/۴ درصد) را روی قارچ بیمارگر داشتند. توانایی عوامل بیوکنترل در تولید سیدروفور، پروتئاز، سیانید هیدروژن و اندول استیک اسید نیز متفاوت بود. نتایج آزمایشات گلخانه‌ای ۷۵ روز بعد ازکشت نشان داد که تمام عوامل بیوکنترل سبب کاهش شدت بیماری و تغییر رنگ آوند در گیاهان تیمار شدند. جدایه *T. flavus* TFPV24 بیشترین تاثیر را در کاهش شدت بیماری (۷۶٪) و تغییر رنگ آوند داشت و باعث افزایش محصول (۱۷۱٪) در مقایسه با شاهد گردید. بطور کلی جدایه های *T. flavus* در شرایط آزمایشگاه و گلخانه بیشترین و باکتری *S. marcescens* SMTR کمترین تاثیر آنتاگونیستی را روی کنترل قارچ بیمارگر داشتند. بنابر نتایج این تحقیق جدایه‌های *T. flavus* توانایی مهار زیستی بیماری پژمردگی ورتیسیلیومی سیب‌زمینی را دارند.

واژه‌های کلیدی: پژمردگی سیب زمینی، کنترل بیولوژیک، *Talaromyces flavus*، *Fusarium oxysporum*.

✉ **Corresponding author: jamini@uok.ac.ir**

Introduction

Potato (*Solanum tuberosum* L.) is the fourth most important food crop in the world after wheat, rice and corn (Desjardins *et al*., 1995) and its products are known to be the most important source of food for human beings (Kotan *et al*., 2009b). The world production of potato is 321 million tonnes in 22 million hectare. Approximately 3.5 million tonnes of potatoes are produced in 140000 hectares of land in Iran (Naraghi *et al*., 2010).

Verticillium wilt is a worldwide important vascular wilt disease caused by *V. dahliae* (Kleb). It is a soil borne pathogen and causes problems in a wide range of herbaceous and woody plant hosts such as potato, strawberry, cauliflower, lettuce, cotton, olive and spinach (Bilodeau *et al*., 2012; Iakovos *et al*., 2009; Olesen *et al*., 2014). *V. dahliae* produces microsclerotia that can survive in the soil for more than 10 years in the absence of a suitable host as resting structures (Olesen *et al*., 2014). Microsclerotia germinate in response to plant root exudates and colonize the root cortex and invades the plant through the roots and then spread to plants vascular system (Johnson *et al*., 2013; Yangui *et al*., 2010). *V. dahliae* is a major cause of potato disease which leads to serious losses in potato fields in Iran and other countries (Aminae *et al*., 2006). It is most severe in irrigated fields, especially in warm climates (Uppal *et al*., 2008). The sclerotia of pathogen are accumulated in the soil from season to season. Therefore, strategies targeting either sclerotia are used to control potato verticillium wilt. Verticillium wilt is difficult to control, because few fungicides could achieve effective dosage to inhibit or even kill the pathogen in ecological niche or in the xylem (Erdogan and Benlioglu, 2010; Yang *et al*., 2013). Management strategies of disease are mainly focused on the use of resistant hosts and cultural practices, but are not always available or effective (Thnassoulopoulos and Hooker, 1968). Biological control is an alternative and has potential for the management of various soil-borne plant pathogens because it is based on the management of a natural resource. Previous studies have shown that biological control by fungi and rhizobacteria can be used successfully to control *V. dahliae* in plants (Erdogan and Benlioglu, 2010; Naraghi *et al*., 2010; Olesen *et al*., 2014; Yang *et al*., 2013; Yangui *et al*., 2010). Plant growth promoting rhizobacteria (PGPR) strains have been shown to be effective biocontrol agents of a number of plant pathogen including *Fusarium solani* (Kotan *et al*., 2009a) and *F. oxysporum* f. sp. *lycopersici* (Abo-Elyousr and Hashem, 2009). *Pseudomonas* spp. are one of the most promising groups of rhizospheric inhabitants which are able to control pathogenic soil-borne microorganisms and show antagonistic activity against diverse phytopathogens (Leticia *et al*., 2009; Tabarraei *et al*., 2011). *Trichoderma* spp. are considered to be antagonistic to soil borne fungi including *Rhizoctonia* spp., *Sclerotina* spp., *Pythium* spp., and *Fusarium* spp. (Bae and Knudsen, 2007). The other research indicated that *Talaromyces flavus* decreased potato Verticillium wilt caused by *V. dahliae* and *V. albo-atrum* (Madi *et al*., 1997; Tjamos and Fravel, 1997).

Biocontrol agents can protect plants from pathogens by different mechanisms, including production of antimicrobial metabolites (Ahmadzadeh and Sharifi Tehrani, 2009), competition for nutrition and space (Tanaka and Omura, 1993), competition for iron through production of siderophores (Sadeghi *et al*., 2012), induction of systemic resistance (Amini and Dzhalilov, 2010), production of extracellular enzymes such as chitinases (Srividya *et al*., 2012) and parasitism (Loliam *et al*., 2013). However, few specialized studies have been conducted concerning biocontrol mechanism and efficacy of biocontrol agents against *Verticillium* wilt of potato. Due to limitation of fungicides application against soil borne pathogens, an effective and convenient method for the application of biocontrol agents against potato verticillium wilt is also needed.The objective of this study was to evaluate the efficacy of some biocontrol agents against *V. dahliae*, the causal agent of potato verticillium wilt under *in vitro* and greenhouse conditions. This research was conducted as a first step toward the development of effective biological control by selected biocontrol agents as an alternative strategy for the management of potato verticillium wilt in Iran.

Materials and Methods

1. Plant material and growth conditions: Potato (*Solanum tuberosum* L.) cultivars Agria susceptible to

Verticillium wilt was used in this research. Healthy potato tubers (40-50 g) having 2-3 eyes (buds) were selected and stored at 2-4°C until used. Seed tubers of potato were soaked in gibberellic acid (GA3) at 1,500 ppm for 24 h to break dormancy and sown in pots (30 cm diameter and 20 cm high) containing pasteurized soil-sand-peat-perlite mix (4:4:4:1, w/w/w/w). Pots were maintained in a greenhouse at 22 to 28°C, 60-70% relative humidity, 16 h light and 8 h darkness. Plants were watered twice a week with sterile tap water and once a week with the fertilizer solution.

2. Pathogen and biocontrol agents: *V. dahliae* was isolated from the infected potato in Ghorveh area in Kurdistan Province, Iran. Pathogen was cultured on potato dextrose agar (PDA, Merk, Germany) at 24-26°C for two weeks and purified by single spore method. Identification of the fungus was done based on morphological and microscopic observation of the forms of colonies, conidia, conidiophore and microsclerotia of *V. dahliae* (Issac, 1967). The purified and identified cultures of *V. dahliae* were stored at 4°C for further use. Conidial suspensions of the pathogen (*V. dahliae*) were prepared by pouring sterile distilled water into PDA plates containing fungal cultures. Two hundred microliter aliquots of conidial suspensions of the pathogen were transferred to flasks (250 ml) containing 150 ml of sterile potato dextrose broth medium. The flasks were shaken at 150 rpm at 25 ± 2°C for 7 days. Culture was filtered through sterile glass wool and then conidial suspension was diluted to a concentration of approximately 1×10^6 spore ml^1.

Source and characteristic of biocontrol agents used in this study are shown in Table 1.

For preparation of antagonistic fungal spore (*T. harzianum*, *T. deliquescens*, *F. oxysporum* and *T. flavus*), at first the fungal isolates were grown on PDA medium for three weeks at 25 ± 2°C. The spores of fungi in the Petri plates were washed out by adding 15 ml of distilled water to each plate. The density of spores was determined by a haemocytometer and then concentration of the suspension was adjusted to 1×10^6 spore ml^{-1}. In addition, for the preparation of the bacterial suspensions (*B. subtilis*, *P. fluorescens* and *S. marcescens*), bacterial isolates were streaked onto Petri plates containing nutrient agar medium (NA) and maintained at 25 ± 2°C with a photoperiod of 12 h. After 48 h, 50 µl of the bacterial isolates were transferred, with a platinum loop, to flasks containing nutrient broth medium. The flasks were shaken at 150 rpm at 25 ± 2°C for 72 h. Then suspension of bacteria was diluted to a concentration of approximately 1×10^8 cfu/ml.

Table 1. Source and characteristic of fungi and bacteria used in this study

Fungal species	Isolate	Source
Trichoderma harzianum	34 (CECT 2413) and 171	University of Buali Sina, Iran
Trichoderma deliquescens	11	University of Buali Sina, Iran
Fusarium oxysporum	Avr5	Moscow Timiryazev Agricultural Academy
Talaromyces flavus	(TFPV36, TFPV24, TFPV45)	Dept. of Plant Protection, Islamic Azad University, Science and Research Branch, Iran
Bacillus subtilis	(B1 and B2)	Dept of Plant Protection, University of Tehran, Iran
Pseudomonas fluorescens	(AP33, CW2, CHAO, PFT14)	Moscow Timiryazev Agricultural Academy
Serratia marcescens	SMTR	Moscow Timiryazev Agricultural Academy

3. Dual culture method: The isolates of biocontrol agents were evaluated against *V. dahliae* in a laboratory by dual-culture method on PDA media. Plates (90 mm diameter) containing PDA were inoculated with 5 mm diameter mycelial disc of 5 days - old culture of *V. dahliae* and fungal biocontrol agents (*T. harzianum* 34, *T. harzianum* 171, non-pathogenic strains of *F. oxysporum*, *T. deliquescens* and *T. flavus*) at equal distance from the periphery. Furthermore, the ability of antagonistic activity of bacterial biocontrol agents (*B. subtilis*, *P. fluorescens* and *S. marcescens*) were tested *in vitro* by dual-culture based on the Erdogan and Benlioglu (2010) method. For this work, five µl of each bacterial biocontrol agent (10^8 cfu/ml) was placed on the plates. After 48 h incubation at 28 °C, a single 5-mm-

diameter mycelial disc was placed in the center of the plates. All plates were incubated at 26 ± 2°C. After 7 days the percentage of growth inhibition (zone of inhibition) was recorded using the following formula: [(diameter in control plate – diameter in treated plate)/ diameter in control plate]×100.

4. Production of volatile metabolites: 100 µl of bacterial biocontrol agents suspension (1×10^8 cfu/ml, 3-days-old culture) and a single 5-mm-diameter mycelial disk of fungal biocontrol agents were placed at the center of Petri dish (90 mm diameter) containing PDA media, and a 5 mm disk of a 5-days- old of pathogen was placed at the center of another Petri dish containing PDA. Both half Petri dishes were placed face to face preventing any physical contact between the pathogen and the biocontrol agents. The pairs of each Petri dish were sealed together with parafilm. Biocontrol agents were replaced with sterile water in control Petri dish. All Petri dishes were incubated at 28°C for 7 days (Karimi *et al*., 2012; Kazempour, 2004). The percentage of growth inhibition was measured as mention above.

5. Production of non-volatile metabolites: The effect on non-volatile metabolites produced by biocontrol agents was determined by the methods of Kraus and Loper (1990). A 0.2 µm cellophane membrane was placed on PDA plates and 200 µl of bacterial biocontrol agents suspension (1×10^8 cfu/ml, 3-days-old culture) and a single 5-mm-diameter mycelial disk of fungal biocontrol agents were placed at the center of plates. Plates were incubated at 28°C. After 72 h, membrane with biocontrol agents growth was removed and 5 mm disk of a pure culture of pathogen (5-days- old) was placed in the center of the Petri dishes. Biocontrol agents were replaced with sterile water in control plates. Percentage of inhibition was determined as mention above.

6. Siderophore production: Firstly, King's medium B agar (KB) and PDA medium containing 0, 25, 50, 100, 1000, mMol of $FeCl_3$ was prepared. Then, 100 µl of bacterial biocontrol agents and a single of 5-mm-diameter mycelial disk of fungal biocontrol agents were cultured in the center of a plate in KB and PDA medium, respectively. Plates were incubated at 28˚C for 5 d. Then, 200 µl of *Geotrichum candidum* (1×10^6 cfu/ml) was sprayed on the plates for detect $FeCl_3$. Then plates were incubated at 28˚C for 72 h. Clear zones surrounding the biocontrol agents colonies suggested siderophpre production by biocontrol agents that caused inhibition of mycelial growth of *G. candidum* (Weller and Cook, 1983).

7. Protease production: Biocontrol agents were evaluated for production of protease by growing them on skim milk agar (SKM) (Chantawannakul *et al*., 2002). Plates were incubated at 28˚C for 24 h. An ability to clear the SKM suspension in the agar was taken as evidence of the secretion of protease.

8. Hydrogen cyanide production: Production of hydrogen cyanide was determined by growing bacterial and fungal biocontrol agents on nutrient agar (NA) and PDA medium at 28˚C for 48 h in plates, respectively. Then, a sterilized filter paper was soaked in 0.5% (w/v) picric acid in 1% Na_2Co_3 and placed on the upper lid of the plates. The plates were sealed with parafilm and incubated at 28˚C for 4 days. A change in the color of the filter paper from yellow to reddish brown was accepted as an index for cyanogenic activity. Non inoculated plates with biocontrol agents were used as control (alstrom, 1987).

9. Indole acetic acid production (IAA): Biocontrol agents were inoculated in nutrient broth (g/l: peptone, 5g; yeast extract, 1.5g; beef extract, 1.5g; and NaCl) with or without tryptophan (500 mg/l) and incubated at 30°C for 5 d (Alstrom 1987). A 5-ml culture was removed from each tube and centrifuged at 10,000 rpm for 15 min. An aliquot of 2 ml supernatant was transferred to a fresh tube to which 100 µl of 10 mM orthophosphoric acid and 4 ml of reagent (1 ml of 0.5 M FeCl3 in 50 ml of 35% HClO4) were added. The mixture was incubated at room temperature for 25 min, and the absorbance of developed pink color was read at 530 nm using a spectrophotometer (Bric *et al*., 1991).

10. Pathogenicity test: For pathogenicity test, tubers of potato c.v Agria at first were superficially disinfested with a solution of 10% sodium hypochlorite, for 3 min and rinsed abundantly with sterile distilled water. Then, potato tubers were dipped into a conidial suspension (10^6 spores/ml) of *V. dahliae* for 30 min and were sown in pots. Control treatment was treated with sterile distilled water. The results of pathogenicity test were calculated two months after inoculation (Spink and Rows, 1989).

11. Biocontrol of potato Verticillium wilt under greenhouse conditions: Bacterial and fungal antagonists were cultured on PDA medium for five and 10 days, respectively. Fifty milliliter of each suspension (10^6-10^8 cfu/ml) of fungal and bacterial antagonist was added to 2 Kg of sterile soil in pots before planting. After two days, potato tubers were dipped into a conidial suspension (10^6 spores/ml) of *V. dahliae* for 30 min. Next, tubers dried under a laminar flow hood, sown in pots consisting of antagonists and then pots were transferred to the greenhouse at 22 to 28^{o}C, 60-70% relative humidity, 16 h light and 8 h darkness. Experiments were designed as completely randomized (CRD) with five replicates per treatment (each replication consists of one pot with two tubers).

12. Disease severity assessment: Disease severity was recorded two months after planting. All plants were rated for wilt symptoms on the scales of 0-3, where 0, no visible symptoms; 1, some chlorosis in older leaves; 2, general chlorosis associated with some necrosis and wilting; 3, severe wilting or death (Spink and Rows, 1989).

In addition, severity of vascular discoloration of potato stems was evaluated by the following scale based on the stem cross-section showing a vascular discoloration: 0, no vascular discoloration; 1, trace to less than 9% of the stem cross-section showing a vascular discoloration; 2, 10-24% of the stem cross-section with a vascular discoloration; 3, 25-49% of the stem cross-section showing vascular discoloration; 4, 50-74% of the stem cross-section exhibiting vascular discoloration and 5, 75-100% of the stem cross-section displaying vascular discoloration (Uppal *et al*., 2008). At potato harvesting time, in order to assess the effect of each biocontrol treatment on yield, tubers in each pot were gathered from each plant, cleaned from soil particles and weighted.

13. Statistical analysis: Experiments were designed in completely randomized design (CRD). All analyses were conducted using the statistical analysis software system (SAS institute, Inc., 2003). The means were compared by Duncan multiple-ranges test (DMRT) at $P \leq 0.05$.

Result and Discussion

1. Pathogenicity test: Results of the pathogenicity test showed that the isolate of *V. dahliae* was able to cause symptom of diseases. Some leaves turned brown and yellow; chlorosis and necrosis of lower leaves are usually the first symptoms of Verticillium wilt, where they can occur on one or both sides of the leaf or the whole potato plant. The disease index of potato wilt in the pathogenicity test was expressed as 2.7 which represent severe wilting or death. Discoloration of the vascular tissue was observed when plant samples were cut longitudinally and plants were stunted under severe infestation. Moreover, symptoms on tubers appeared as brown discoloration of the vascular ring. To confirm Koch‘s postulates, *V. dahliae* was successfully re-isolated from the inoculated plants.

2. The growth inhibitory effects on *V. dahliae* in three investigations (dual culture, volatile and non-volatile compounds): All biocontrol agents except *F. oxysporum* Avr5 and *S. marcescens* exhibited more than 50% inhibition of mycelia growth of *V. dahliae* by using dual-culture method. The maximum percent inhibition of growth of *V. dahliae* was observed by *T. flavus* isolate TFPV45 (66.7%) followed by isolates *T. flavus* TFPV36, *P. fluorescens* AP33, *T. harzianum* 34, *T. flavus* TFPV24 and *T. harzianum* 171 (ranging from 60% to 65.4%) (Table 2). Inhibitory effects on *V. dahliae* growth in volatile test induced by different biocontrol agents varied (Table 2). The maximum percentage of growth inhibition of *V. dahliae* was observed by *P. fluorescens* AP33 (79%) and minimum inhibitory effect by *T. flavus* TFPV45 (14%). In addition, results of the inhibitory effects by non-volatile test on *V. dahliae* growth showed that nine biocontrol agents exhibited a more than 50% inhibition of mycelia growth of *V. dahliae*. The maximum percentage of growth inhibition of the pathogen was belonged to *T. flavus* TFPV45 (77%) (Table 2).

3. Production of antifungal metabolites: Results of *in vitro* test by 14 biocontrol agents against the pathogen showed that biocontrol agents exhibited different combinations of antimicrobial metabolites such as: siderophore, protease, hydrogen cyanide and IAA (Table 2).

However, only isolates of *P. fluorescens* inhibited *G. candidum* spores germination and produced siderophore (Table 2). All isolates of *P. fluorescens* produced clear zones

around itself that suggested siderophore production in the presence of 25, 50, 100 and 1000 mMol $FeCl_3$. Clear zones of inhibition against *G. candidum* decreased as the concentration of $FeCl_3$ in the King' s medium B agar was increased from 0, 25, 50, 100, 1000 mMol. Production of antimicrobial metabolites (siderophore, protease, hydrogen cyanide and indole acetic acid) was not observed by isolates of *T. flavus* (Table 2).

4. Inhibition of *V. dahliae* by biocontrol agents under greenhouse condition: Results of the antagonistic effects on *V. dahliae* under greenhouse conditions indicated that all biocontrol agents displayed differences in efficiency of suppressing disease. All treatments significantly decreased both the disease severity and browning of vascular tissues in lower stem section and increased fresh weights of tubers compared to untreated control (pathogen alone) (Table 3). Among the 14 biocontrol agents, *T. flavus* TFPV24, *T. flavus* TFPV36 and *T. flavus* TFPV45 were the most effective antagonists against *V. dahliae* and reduced severity of disease by 76%, 68% and 60%, respectively. These isolates increased fresh weight of tubers by more than 100% and reduced severity of vascular discoloration of potato stems 66 to 76%. Minimum inhibitory effect was mediated by isolates of *S. marcescens* (Table 3).

The present study was conducted to evaluate the efficacy of several biocontrol agents against potato Verticillium wilt. The results of this study show that isolates of *T. flavus* have significant antagonistic effects on *V. dahliae*, a pathogenic fungus causing potato verticillium wilt. The effects of biocontrol agents were first evaluated against *V. dahlia*e in *in vitro* conditions (i.e., dual culture assay, volatile and non-volatile metabolites tests). Results illustrated that the isolates of *T. flavus*, caused significant reduction in *V. dahliae* growth through non-volatile and volatile metabolites production mechanisms *in vitro* conditions respectively. Furthermore, these strains showed high efficiency *in vitro* as inhibition zones in dual-culture assay, volatile metabolites, and non-volatile metabolites. The metabolites which produced by these biocontrol agents have a direct role to play in reduction of different soil-borne fungal pathogens in potato (Naraghi *et al*., 2010; Tariq *et al*., 2010). This result is in agreement with other authors that have shown, *Pseudomonas* spp. inhibited the growth of *V. dahliae in vitro* in cotton (Erdogan and Benlioglu, 2010; Yang *et al*., 2013), *R. solani* in potato (Tariq *et al*., 2010), *Phytophthora drechsleri* in cantaloupe (Tabarraei *et al*., 2011) and *Fusarium oxysporum* f. sp. *ciceris* in chickpea (Karimi *et al*., 2012).

Table 2. *In vitro* activity of biocontrol agents against *Verticillium dahliae* and production of antimicrobial metabolites.

Treatments	DC(%)	Va(%)	NVa(%)	ST	PT	HC*	IAA
Trichoderma harzianum 34	62 ^{D}	53.47 ^{G}	73.70 ^{C}	-	+	-	+
Trichoderma harzianum 171	60.02 ^{E}	71.00 ^{B}	55.70 ^{G}	-	+	-	-
Trichoderma deliquescens 11	50.00 ^{J}	47.00 ^{I}	63.00 ^{F}	-	+	-	-
Fusarium oxysporum Avr5	41.05 ^{L}	21.00 ^{M}	29.00 ^{I}	-	-	-	-
Talaromyces flavus TFPV36	65.37 ^{B}	34.00 ^{K}	78.00 ^{A}	-	-	-	-
Talaromyces flavus TFPV24	60.00 ^{E}	57.02 ^{F}	69.00 ^{D}	-	-	-	-
Talaromyces flavus TFPV45	66.65 ^{A}	14.07 ^{N}	77.00 ^{B}	-	-	-	-
Bacillus subtilis B1	47.37 ^{K}	53.00 ^{H}	24.00 ^{J}	-	+	-	+
Bacillus subtilis B2	51.10 ^{I}	68.40 ^{D}	19.00 ^{M}	-	+	-	+
Pseudomonas fluorescens AP33	64.00 ^{C}	79.00 ^{A}	63.30 ^{E}	+	+	++	+
Pseudomonas fluorescens CW2	52.07 ^{H}	69.40 ^{C}	63.02 ^{F}	+	+	+++	+
Pseudomonas fluorescens CHAO	55.07 ^{G}	62.00 ^{E}	51.00 ^{H}	+	+	+	+
Pseudomonas fluorescens PFT14	58.97 ^{F}	39.00 ^{J}	20.30 ^{L}	+	-	+	+
Serratia marcescens SMTR	31.45 ^{M}	28.00 ^{L}	23.00 ^{K}	-	-	-	-
Non infested control	0.00 ^{N}	0.00 ^{O}	0.00 ^{N}	-	-	-	-

Means in the column followed by different letter's indicate significant differences among treatments at $P \leq 0.05$ according to Duncan multiple-ranges test (DMRT). Data are means of four replicates. DC = percent growth inhibition in dual culture method, VA = percent growth inhibition in Volatile metabolites, NVa = percent growth inhibition in Non- Volatile metabolites, ST= Siderophore test, PT = Protease test, HC = Hydrogen cyanide, IAA = indole acetic acid. *, +: present, -: absent, +++: enhanced activity.

Table 3. Effect of biocontrol agents on *V. dahliae* and potato yield under greenhouse conditions after 75 days

Treatments	DS	DR%	VD	TFW (g)	Increase%
Non infested control	0.00 J	-	0.00 A	378.00 A	-
Control (pathogen)	2.50 A	-	2.10 K	118.00 L	-
Trichoderma harzianum 34	1.75 D	30.0	1.30 H	172.25 H	45.97
Trichoderma harzianum 171	1.50 E	40.0	0.90 E	194.00 F	64.40
Trichoderma deliquescens 11	1.07 G	57.2	0.80 D	277.25 C	134.95
Fusarium oxysporum Avr5	1.25 F	50.0	1.20 G	190.25 F	61.22
Talaromyces flavus TFPV36	0.80 H	68.0	0.50 B	321.25 B	172.24
Talaromyces flavus TFPV24	0.60 I	76.0	0.50 B	320.00 B	171.18
Talaromyces flavus TFPV45	1.00 G	60.0	0.70 C	257.75 D	118.43
Bacillus subtilis B1	2.00 C	20.0	1.87 J	140.50 I	19.06
Bacillus subtilis B2	2.20 B	12.0	1.77 I	143.25 I	21.39
Pseudomonas fluorescens AP33	1.40 E	44.0	0.90 E	200.00 E	69.49
Pseudomonas fluorescens CW2	2.02 C	19.2	1.70 I	134.00 J	13.56
Pseudomonas fluorescens CHAO	1.72 D	31.2	1.00 F	181.50 G	53.81
Pseudomonas fluorescens PFT14	2.02 C	19.2	1.87 J	126.00 K	6.78
Serratia marcescens SMTR	2.30 B	8.0	2.02 K	118.00 6L	0.00

Means in the column followed by different letter's indicate significant differences among treatments at $P \leq 0.05$ according to Duncan multiple-ranges test (DMRT). Data are means of five replicates. DS = Disease severity, DR = Disease reduction, VD = Vascular discoloration, TFW = Tubers fresh weights.

Potato verticillium wilt was reduced 60-76% by *T. flavus* isolates in comparison with the untreated control. Isolates of *T. flavus* were the most effective fungal antagonist in both laboratory and greenhouse conditions. The effect of volatile and non-volatile extracts of *T. flavus* on sugar beet damping-off caused by *Rhizoctonia solani* and root rot disease of lettuce caused by *Sclerotinia minor* have been demonstrated *in vitro* and greenhouse conditions (El-Tarabily *et al*., 2000; Saeed *et al*., 2013) which is in agreement with the results of this research. Furthermore, results of another study indicated that non-volatile extract such as chitinase enzyme produced by *T. harzianum* and *T. flavus* were effective on *S. sclerotiorum* and *S. rolfsii* in soybean stem white rot and bean stem rot disease, respectively (Madi *et al*., 1997; Menendez and Godeas, 1998).

Results of our greenhouse studies indicated that strains of *T. flavus* were the most important antagonist of *V. dahliae*, which demonstrated significant effects on reducing disease severity, vascular discoloration of Verticillium wilt and increasing of weight of fresh tubers compared to the untreated control (pathogen alone). Results of *in vitro* test indicated that antimicrobial metabolites such as siderophore, protease, hydrogen cyanide and indole acetic acid were not produced by *T. flavus* isolates. Therefore, the results of greenhouse experiments indicated that the other metabolites (volatile and non-volatile metabolites) produced by these biocontrol agents have a direct role in reduction of disease. Isolates of *T. flavus* has been applied as a biocontrol agent, a producer of secondary metabolites or enzymes (Bohumil Proksa, 2010). It has been shown as an important antagonist against *V. albo-atrum* (Naraghi *et al*., 2010), because this antagonist produced glucose oxidas (Kim *et al*., 1990) and 2-methyl sorbic acid (Proksa *et al*., 1992) which inhibited formation of microsclerotia of *V. dahliae* and growth of *V. albo-atrum*, respectively. 3-hydroxymethl-6, 8-dimethoxycoumarin was isolate from a liquid non-agitated culture of *T. flavus* on malt extract medium (Ayer and Racok, 1990). Biological control of *V. dahliae* and *Sclerotium rolfsii* by *T. flavus* is mediated by different mechanisms including mycoparasitism (chitinase activity), production of antifungal compounds, glucose-oxidase activity, melanization of newly formed microsclerotia and plant root colonization (Fahima *et al*., 1990; Madi *et al*., 1997). In addition, *T. flavus* antagonizes *V. dahliae* by parasitism and antibiosis (Fahima *et al*., 1992; Marois *et al*., 1984). Chitinase activity was decomposed the cell wall of *Verticillium dahlia*, *Sclerotinia*

sclerotiorum and *Rhizoctonia solani* (Dou-Chuan *et al*., 2005). Mycelium and microsclerotia of *V. dahliae* were very sensitive to the antibiotic produced by *T. flavus*. These antibiotic inhibited melanization of newly formed *V. dahliae* microsclerotia and the prevention of microsclerotial melanization could affect their survival in soil and make microsclerotia sensitive to antagonistic microorganisms (Fahima and Henis, 1995; Madi *et al*., 1997; Tjamos and Fravel, 1995).

This study has shown that selected biocontrol agents were effective in inhibiting *V. dahliae*. Our results demonstrate that isolates of *T. flavus* were the most effective and produced a higher level of inhibition of pathogen *in vitro* and under greenhouse conditions. Therefore, they have great potential to be used as biocontrol agents and could be a viable strategy for controlling potato Verticillium wilt in the field as practical application. It recommend that application of biocontrol can lead to beneficial results and provide significant protection against the potato Verticillium wilt through potato root colonization. Application of this microbe for diseases management and their practical use requires further investigation under field conditions.

Acknowledgments

The author is thankful to the vice Chancellor for research of University of Kurdistan for the financial support, (Grant No. 1/27238).

References

ABO-ELYOUSR, K. A. and M. M. HASHEM, 2009. Biological control of Fusarium wilt in tomato by plant growth-promoting yeasts and rhizobacteria. Plant Pathology Journal, 25(2): 199-204.

AHMADZADEH, M. and A. SHARIFI TEHRANI, 2009. Evaluation of fluorescent Pseudomonads for plant growth promotion, antifungal activity against *Rhizoctonia solani* on common bean, and biocontrol potential. Biological Control, 48: 101-107.

ALSTROM, S. 1987. Factors associated with detrimental effects of rhizobacteria on plant growth. Plant soil, 102: 3-9.

AMINAE, M. M., B.MANSOORI and D. ERSHAD, 2006. A study on Verticillium wilt of Potato in Kerman province. In Proceedings of the 17th Iranian plant protection Congress, 163 University of Tehran, Karaj.

AMINI, J. and F. DZHALILOV, 2010. Induction of systemic resistance in tomato plants to *Fusarium oxysporum* f. sp. *lycopersici* causal agent of Fusarium wilt of tomato by non-pathogenic *F. oxysporum* under greenhouse conditions. Applied Entomology and Phytopathology, 78(1): 15-32.

AYER, W. A. and J. S. RACOK, 1990. The metabolites of *Talaromyces flavus*. Part 1. Metabolites of organic extract. Canadian Journal of Chemistry, 68: 2085-2094.

BAE, Y. S. and G. R. KNUDSEN, 2007. Effect of sclerotial distribution pattern of *Sclerotinia sclerotiorum* on biocontrol efficacy of *Trichoderma harzianum.* Applied Soil Ecology, 35(1): 21-24.

BILODEAU, G. J., S. T. KOLIKE, URIBE, P. and F. N. MARTIN, 2012. Develiopment of an assay for rapid deterction and quantification of *Verticillium dahlia* in soil. Phytopathology, 102: 331-343.

BOHUMIL PROKSA. 2010. *Talaromyces flavus* and its metabolites. Chemical Papers, 64(6): 696-714.

BRIC, J. M., R. M. BOSROCK and S. E. SILVERSONE, 1991. Rapid in situ assay for indole acetic acid production by bacteria immobilization on a nitrocellulose membrane. Applied and Environmental Microbiology, 57: 535-538.

CHANTAWANNAKUL, P., A. ONCHAROEN, K. KLANBUT, E. CHUKEATIROTE and S. LUMYONG, 2002. Characterization of proteases of *Bacillus subtilis* strain 38 isolated from traditionally fermented soybean in northern Thailand. Science Asia, 28: 241-245.

DESJARDINS, A. E., S. P. MCCORMICK and D. L. CORSINI, 1995. Diversity of sesquiterpenes in 46 potato cultivars and breeding selections. Journal of Agricultural and Food Chemistery, 43: 2267-2272.

DUO-CHUAN, L. I., S. CHEN and L. U. JINA, 2005. Purification and partial characterization of two chitinases from the mycoparasitic fungus *Talaromyces*

flavus. Mycopathologia, 159: 223-229.

EL-TARABILY, K. A., M. H. SOLIMAN, A. H. NASSER, H. A. AL-HASSANI, K. SIVASITHAMPARAM, F. MCKENNA and G. S. HARDY, 2000. Biological control of *Sclerotinia minor* using a chitinolytic bacterium and actinomycetes. Plant Pathololgy, 49(5): 573-583.

ERDOGAN, O. and K. BENLIOGLU, 2010. Biological control of Verticillium wilt on cotton by the use of fluorescent *Pseudomonas* spp. Under field conditions. Biological Control, 53: 39-45.

FAHIMA, T. and Y. HENIS, 1995. Quantitative assessment of the interaction between the antagonistic fungus *Talaromyces flavus* and the wilt pathogen *Verticillium dahliae* on eggplant roots. Plant soil, 176(1): 129-137.

FAHIMA, T., Y. HENIS and D. HORNBY, 1990. Interactions between pathogen, host and biocontrol agent: multiplication of *Trichoderma hamatum* and *Talaromyces flavus* on roots of diseased and healthy hosts. In Biological control of soil-borne plant pathogens, 165-180: CAB International.

FAHIMA, T., L. MADI and Y. HENIS, 1992. Ultrastructure and germinability of *Verticillium dahliae* microsclerotia parasitized by *Talaromyces flavus* on agar medium and in treated soil. Biocontrol Science and Technology, 2: 69-78.

IAKOVOS, S. P., E. T. SOTIRIOS, A. S. IOANNIS, C. IORDANIS and J. P. EPAMINONDAS, 2009. Mode of action of a non-pathogenic *Fusarium oxysporum* strain against *Verticillium dahliae* using real time QPCR analysis and biomarker transformation. Biological Control, 50(1): 30-36.

ISSAC, I. 1967. Speciation in Verticillium. Annu Rev Phytopathol, 5(1): 201-222.

JOHNSON, D. A., R. BAKER and R. A. BOYDSTON, 2013. Field evaluation of mutant and hybrid lines of mint for resistance to Verticillium wilt and yield. Crop protection, 43: 1-6.

KARIMI, K., J. AMINI, B. HARIGHI and B. BAHRAMNEJAD, 2012. Evaluation of biocontrol potential of *pseudomonas* and *bacillus* spp. against Fusarium wilt of chickpea. Aust J Crop Science, 6(4): 695-703.

KAZEMPOUR, M. N. 2004. Biological control of *Rhizoctonia solani*, the causal agent of rice sheath blight by antagonistic bacteria in greenhouse and field conditions. Plant Pathology journal, 3(2): 88-96.

KIM, K. K. A., D. R. FRAVEL and G. C. PAPAVIZAS, 1990. Glucose oxidas as the antifungal principle of talaron from *Talaromyces flavus*. Canadian Journal of Microbiology, 36(11): 760-764.

KOTAN, R., S. FIKRETTIN, D. ERKOL and E. CAFER, 2009a. Biological control of the potato dry rot caused by *Fusarium* species using PGPR strains. Biological Control, 50(2): 194-198.

KOTAN, R., F. SAHIN, E. DEMIRIC and C. EKEN, 2009b. Studies on the biological control of potato dry rot disease caused by *Fusarium solani* with application of some bacterial strains. In 5th Biological control congress Erzuum, Turkey.

KRAUS, J. and J. E. LOPER, 1990. Biocontrol of Pythium damping-off of cucumber by *Pseudomonas fluorescens* pf-5: Mechanistic studies In Plant Growth Promoting Rhizobacter, 172-175 (Eds C. Keel, B. Koller and G. Defago). Interlaken, Switzerland: The second international workshop on plant growth promoting rhizobacteria

LETICIA, Q., A. GASTON, B. NATALIA, V. PATRICIA, P. CARLOS, D. FERNANDO, C. MONICA, A. NORA and A. ALICIA, 2009. Three native *Pseudomon fluorescens* strains tested under growth chamber and field conditions as biocontrol agents against damping-off in alfalfa. Biological Control, 51: 42-50.

LOLIAM, B., T. MORINAGA and S. CHAIYANA, 2013. Biocontrol of *Pythium aphanidermatum* by the cellulolytic actinomycetes *Streptomyces rubrolavendulae* S4. Science Asia, 39(6): 584-590.

MADI, L., T. KATAN, J. KATAN and Y. HENIS, 1997. Biological control of *Sclerotinia rolfsii* and *Verticillium dahlia* by *Talaromyces flavus* is mediated by different mechanisms. Phytopathology, 87: 1054-1060.

MAROIS, J. J., D. R. FRAVEL and G. C. PAPAVIZAS,

1984. Ability of *Talaromyces flavus* to occupy the rhizosphere and its interaction with *Verticillium dahliae*. Soil Biology and Biochemistry, 6: 387-390.

MENENDEZ, A. B. and A. GODEAS, 1998. Biological control of *Sclerotinia sclerotinia* attacking soybean plants: degradation of the cell wall of this pathogen by *Trichoderma harzianum*. Mycopathology, 142: 153-160.

NARAGHI, L., A. HEYDARI, S. REZAEE, M. RAZAVI, and H. JAHANFAR, 2010. Study on antagonistic effect of *Talaromyces flavus* on *Verticillium albo-atrum*, the causal agent of potato wilt disease. Crop protection, 29: 658-662.

OLESEN, M. H., L. C. DELEURAN, R. GISLUM and B. BOELT, 2014. Preventing an increase in Verticillium wilt incidence in spinach seed production. Crop protection, 66: 107-113.

PROKSA, B., J. ADAMCOVA and J. FUSKA, 1992. 2-Methylsorbic acide, an antifungal metabolite of *Penicillium vermiculatum*. Journal Applied Microbiology and Biotechnology, 37: 443-445.

SADEGHI, A., E. KARIMI, P. A. DAHAJI, M. G. JAVID, Y. DALVAND and H. ASKARI, 2012. Plant growth promoting activity of an auxin and siderophore producing isolate of *Streptomyces* under saline soil conditions. World Journal of Microbiology and Biotechnology, 28(4): 1503-1509.

SAEED, R., N. KAKVAN, A. HEYDARI, L. NARAGHI, and H. R. ZAMANIZADEH, 2013. Development of new bioformulation using *Trichoderma* and *Talaromyces* fungal antagonists for biological control of sugar beet damping-off disease. Crop protection, 53: 80-84.

SPINK, D. S. and R. C. ROWS, 1989. Evaluation of *Talaromyces flavus* as abiological control agent against *Verticillium dahliae* in potato. Plant Disease, 73: 230-236.

SRIVIDYA, S., A. THAPA, D. V. BHAT, K. GOLMEI and N. DEY, 2012. *Streptomyces* sp. 9p as effective biocontrol against chilli soilborne fungal phytopathogens. Eurropean Journal of Experimental Biology, 2(1): 163-173.

TABARRAEI, M., J. AMINI and B. HARIGHI, 2011. Effects of fluorescent Pseudomonads for control of damping-off disease of cantaloupe caused by *Phytophthora drechsleri*. Australian Journal Crop Science, 5(11): 1427-1433.

TANAKA, Y. and S. OMURA, 1993. Agroactive compounds of microbial origin. Annual Review of Microbiology, 47(1): 57-87.

TARIQ, M., S. YASMIN and F. Y. HAFEEZ, 2010. Biological control of potato black scurf by rhizosphere associated bacteria. Brazilian Journal of Microbiology, 41(2): 439-451.

THANASSOULOPOULOS, C. C. and W. J. HOOKER, 1968. Factors influencing infection of field grown potato by *Verticillium albo-atrum*. American Potato Journal, 45: 203-216.

TJAMOS, E. C. and D. R. FRAVEL, 1995. Detrimental effect of sublethal heating and *Talaromyces flavus* on microsclerotial of *Verticillium dahlia*. Phytopathology, 85: 388-392.

TJAMOS, E. C. and D. R. FRAVEL, 1997. Distribution and establishment of the biocontrol fungus *Talaromyces flavus* in soil and on roots of solanaceous crops. Crop protection, 16(2): 135-139.

UPPAL, A, K. and A. E. HADRAMI, R. L. ADAM, M. TENUTA and F. DAAY, 2008. Biological control of potato Verticillium wilt under controlled and field conditions using selected bacterial antagonists and plant extracts. Biological Control, 44: 90-100.

WELLER, D. M. and R. J. COOK, 1983. Suppression of take all of wheat by seed treatment with fluorescent pseudomonads. Phytopathology, 73: 463-469.

YANG, P., Z. X. SUN, S. Y. LIU, H. X. LU, Y. ZHOU and M. SUN, 2013. Combining antagonistic endophytic bacteria in different growth stages of cotton for control of Verticillium wilt. Crop prottection, 47: 17-23.

YANGUI, T., S. SAYADI, A. GARGOUBI and A. DHOUIB, 2010. Fungicidal effect of hydroxytyrosol-rich preparation from olive mill wastewater against *Verticillium dahlia*. Crop protection, 29: 1208-1213.