doi: 10.17221/55/2016-PPS

Potential of Some Bacteria for Biological Control of Postharvest Citrus Green Mould Caused by *Penicillium digitatum*

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Abstract

Mohammadi P., Tozlu E., Kotan R., Kotan Şenol M. (2017): Potential of some bacteria for biological control of postharvest citrus green mould caused by *Penicillium digitatum*. Plant Protect. Sci., 53: 134–143.

Ten bacteria isolate (4 *Bacillus subtilis*, 2 *Bacillus pumilus*, 2 *Bacillus cereus*, 1 *Bacillus megaterium*, and 1 *Agrobacterium radiobacter*) were tested *in vitro* for antagonistic properties against *Penicillium digitatum*, the causal agent of citrus green mould. The effect of these bacteria was also observed on mycelial growth, spore germination, and spore production of the pathogenic fungus in broth culture. Extracellular enzyme activities of the bacteria were determined. According to the results of *in vitro* antagonistic tests and enzymes activities, the most promising bacteria were *Bacillus subtilis* and *Agrobacterium radiobacter*. These bacteria were tested for disease suppression on lemon fruits. In addition, these bacterial isolates also showed remarkable antifungal activity against the pathogen on lemon fruits. The results of this study showed that *Bacillus subtilis* and *Agrobacterium radiobacter* showed remarkable antifungal activity against the pathogen. Chitinase and glucanase enzyme activity of all the tested bacteria was positive. Protease enzyme activity was positive in all tested bacteria with the exception of *Agrobacterium radiobacter*. In addition, all bacteria inhibited mycelial growth and spore germination (except *Agrobacterium radiobacter*) of the fungus. *Bacillus subtilis*, *Bacillus cereus*, and *Agrobacterium radiobacter* inhibited spore production in broth culture. *Bacillus subtilis* and *Agrobacterium radiobacter* were tested on lemon fruits significantly reduced disease severity. Consequently, these isolates can be used as new biocontrol agents in controlling the post-harvest decay of citrus fruits caused by *Penicillium digitatum*.

Keywords: *Bacillus* spp.; biocontrol; citrus green mould; postharvest disease; *Agrobacterium radiobacter*

Citrus is one of the most widely produced fruits globally. It is grown commercially in more than 137 countries around the world (Ismail & Zhang 2004) with worldwide crop production of about 115.525 thousand tons in 2015 (FAO 2015). Citrus fruits can be infected by many fungal pathogens, and these pathogens cause considerable losses during storage and transportation (Maldonado *et al.* 2009). Green mould, caused by the pathogen *P. digitatum*, is the most economically important postharvest disease of citrus fruits in all production areas (Eckert & Eaks 1989). The pathogen is the most devastating one, causing about 90% of production losses during the postharvest handling of fruits (Macarisin *et al.* 2007; Moss 2008; Zamani *et al.* 2009).

The postharvest disease has been primarily controlled worldwide by the application of synthetic fungicides such as imazalil, sodium *ortho*-phenylphenate, thiabendazole pyrimethanil, azoxystrobin and fludioxonil (Schirra *et al.* 2005, 2010; D'Aquino *et al.* 2006; Smilanick *et al.* 2006; Kanetis *et al.* 2007). The fruit surface is completely covered by the fungicides, and their residues remain on the fruit surface (Holmes & Eckert 1999). The increasing concern for health hazards and environmental pollution due to the use of chemicals has required the development

doi: 10.17221/55/2016-PPS

of alternative strategies for the control of postharvest citrus diseases. Management of postharvest diseases using microbial antagonists, natural plant-derived products and compounds that are generally recognised as safe has been demonstrated to be most suitable to replace the synthetic fungicides, which are either being banned or recommended for limited use (Smilanick *et al*. 2005; Sharma *et al*. 2009; Talibi *et al.* 2014).

Biological control is becoming an important alternative in the fight against postharvest diseases of fruits and as a result, there is an urgent need for further research in order to develop new and more efficient strategies for bio-control (Pimenta *et al.* 2008). Successful control of infections caused by a number of postharvest pathogens using antagonistic bacteria has been reported on citrus fruits, including *Paenibacillus brasilensis*, *Bacillus subtilis*, *Burkholderia gladioli* pv. *agaricicola*, and *Streptomyces* sp. (Kotan *et al.* 2009; Elshafei *et al*. 2012; Ketabchi *et al*. 2012; Mohammedi *et al.* 2014).

This study aimed to evaluate the antifungal activities of *B. subtilis* (TV-6F, TV-12H, TV-17C, and BA-140), *B. pumilus* (RK-103 and TV-73F), *B. cereus* (EK-7 and TV-79B), *B. megaterium* (TV-103B), and *A. radiobacter* (A-16) against *P. digitatum* under *in vitro* conditions. Also promising bacteria were tested as biocontrol agents in *in vivo* conditions.

MATERIAL AND METHODS

Tested fruits. In this study, lemon fruits (*Citrus limon* L.) belonging to Meyer cultivars used in the experiments were obtained from a local market. The fruits were selected free of wounds and rots and as homogeneous as possible in size, and were stored at 5°C under dry conditions until use.

Pathogen and growth conditions. The pathogenic fungus *P. digitatum* that had been isolated from a decayed lemon fruit and potential biocontrol bacteria were obtained from the culture collection unit in the Department of Plant Protection, Faculty of Agriculture at Ataturk University, Turkey (Table 1).

Tested bacteria and growth conditions. The biocontrol bacteria had been isolated from the rhizosphere and phyllosphere of wild and traditionally cultivated plants growing in the Eastern Anatolia Region of Turkey (Kotan 1998; Kotan *et al*. 2009; Çakmakçı *et al*. 2010; Tozlu *et al.* 2016). The fungal pathogen was grown on Potato Dextrose Agar (PDA) and maintained on PDA slant cultures at 4°C in the refrigerator. Bacterial cultures were grown on Nutrient Agar (NA) for routine use, and maintained in Nutrient Broth (NB; all Difco, Le Pont de Claix, France) with 15% glycerol at –80°C for long-term storage.

The identity of nine bacterial isolates [*B. subtilis* (TV-6F, TV-12H, TV-17C, and BA-140); *B. pumilus* (RK-103 and TV-73F); *B. cereus* (EK-7 and TV-79B), and *B. megaterium* (TV-103B)] in a previous study and of *A. radiobacter* (A 16) and pathogenic fungus in this study was confirmed according to fatty acid methyl ester (FAME) analysis using Sherlock Microbial Identification System (Microbial ID, Newark, USA) (Miller 1982; Sasser 1990) and BIOLOG systems (Holmes *et al.* 1994). FAMEs were separated by gas chromatography (HP6890; Hewlett Packard, Palo Alto, USA) with a fused-silica capillary column (25 m × 0.2 mm) with cross-linked 5% phenyl methyl silicone. FAME profiles of each bacterial isolate were identified by comparing the commercial databases (TSBA 40) with the MIS software package. BIOLOG identification was performed using BIOLOG420/Databases/GN601 and GP601 KID software.

Hypersensitivity test. The potential biocontrol bacterial isolates were tested for hypersensitivity on tobacco (*Nicotina tabacum* L. var. Samsun) plants as described by Klement *et al.* (1964) in previous studies. The bacterial suspension (1×10^8 CFU/ml) was prepared in sterile distilled water and infiltrated into the intercostal area of the leaves of tobacco plants using a 3-cc syringe without needle (Becton Dickinson, Franklin Lakes, USA). The inoculated plants were incubated in a completely randomized design on the greenhouse bench for 24–48 h at 20–28°C. The presence of rapid tissue necrosis at the inoculation site was recorded within 24–48 h after infiltration. This test was repeated at least three times for each isolate. Sterilised distilled water (sdH_2O) was used as a negative control.

Pathogenicity test. Pathogenicity tests of the antagonistic bacteria and pathogenic fungus were performed on lemon fruits belonging to Meyer cultivars. The cell suspension of the antagonistic bacterial isolates (1×10^8 CFU/ml) and spore suspension (1×10^5 CFU/ml) were supplemented with Tween 20. The lemon fruits were washed under tap water and then surface sterilised with 70% ethanol and washed twice by immersion in distilled and sterilised distilled water. They were left in a dry place to remove excess water on the surface until being used for pathogenicity test. Fruits were dipped into a cell suspension of the antagonistic bacterial cul-

doi: 10.17221/55/2016-PPS

tures and spore suspension of the pathogenic fungus. Afterwards, the fruits were sealed in polyethylene-lined plastic boxes, and they were stored at 10°C, at 80% humidity under a photoperiod of 12-h light and 12-h dark. After incubation for 7 days, decay on the fruit surface and/or fungal mycelial growth or spore germination were determined as a positive pathogenicity test. Distilled water wasalone was used as a negative control. Treatments were arranged in a randomised block design. Each treatment was applied to three replicates of 5 fruits in each experiment.

Estimation of chitinase. Chitinase enzyme activities were determined according to Senol *et al.* (2014). Enzyme solution (0.2 ml) and 0.5 ml 0.5% (w/v) of colloidal chitin (that was prepared in 50 mM citrate buffer pH 6) were incubated at 37°C for 30 minutes. The reaction was stopped by addition of 0.75 ml 3,5-dinitrosalicylic acid (DNS) reagent (Miller 1959), followed by heating at 80°C for 10 minutes. The activity was estimated at 540 nm. One unit of the enzyme activity was defined as the amount of the enzyme that catalysed the release of 1 µmol/ml/min of reducing sugar under the above-mentioned assay conditions.

Estimation of glucanase. Glucanase activity was detected according to the Teather and Wood method (Teather & Wood 1998) using lichenan (0.2%). After incubation at 30°C for 4–5 days, the plates were flooded with specific staining solutions; Coango red 0.3% for glucanase.

Estimation of protease. Determination of the enzyme protease was carried out on plates of Skim Milk Agar (SMA) medium (containing 15 g skim milk, 0.5 g yeast extract, 9.13 g agar, and 1 l distilled water). The plates were inoculated with bacteria and incubated at 27°C for 24 h (Atlas 1997). Diameters of the colourless halo zone around the bacterial colonies were measured to determine the ability of glucanase and protease production.

Screening of bacterial cultures for antifungal activity. The antifungal activity assays included culture filtrates, cell-free filtrates and volatile metabolites of the bacteria tested against *P. digitatum* on PDA medium.

The antifungal activity of the bacterial culture against *P. digitatum* grown on PDA was observed using the following method. The fungus was grown in a PDA plate (90 mm) at 30°C, in a 12 h light/dark cycle seven days before inoculation. The fresh culture of the fungus was cut out with a cork borer (6 mm diameter), and placed in the centre of 90 mm Petri plates with PDA medium. The bacteria were grown in 50-ml Erlenmeyer flasks containing 20 ml of Nutrient Broth (NB) medium on a rotary shaker at 28°C for 24 hours. Dilution to about 1×10^8 CFU/ml of bacterial suspension. Bacterial suspensions were individually streaked with a sterile swap on the Petri plates as a circular inner edge of the plate. The plates were wrapped together with parafilm, and were incubated at 28°C until fungal mycelia completely covered the agar surface in a control plate and the diameters of the fungal colonies were scored and measured in mm.

The antifungal activity of cell-free filtrates of bacteria against fungi grown on PDA was observed using the diffusion method (Mehmood *et al.* 1999). Erlenmeyer flasks containing 150 ml of liquid NB medium were inoculated with 1.5 ml bacterial suspension containing 10^8 CFU/ml and incubated under rotary shaking (180 rpm) at 28°C for 7 days. Then, the culture was centrifuged at 20 000 *g* for 15 min and membrane filtered (0.20 µm; Millipore, Merck KGaA, Darmstadt, Germany), then 10 µl of filtrate was dripped on 14 ml of solid PDA medium inoculated with 6 mm of fungal disk. After 4–5 days of incubation, the diameters of the fungal colonies were scored and measured in mm.

The volatile metabolite assays were carried out with minor modification according to Evmert *et al.* (2007) by plating 100 µl of bacterial suspension containing 10^8 CFU/ml on the surface of NA plates, where the tested fungus was inoculated on PDA plates and the bottom parts of the plates were tightly joined with parafilm and incubated in dark at 8°C for 4–5 days. Fungitoxicity of volatile metabolites was expressed by measuring the diameter of mycelium growth (mm). Control treatments of the experiment consisted of only the pathogenic fungus *P. digitatum* without bacteria. All treatments of the experiment were carried out twice with three replicates.

The antifungal activities of whole bacterial culture, cell-free filtrates and volatile metabolite assessments were determined against *P. digitatum* and fungitoxicity was expressed as the percentage of growth inhibition calculated according to Mari *et al.* (1993) formula:

$$\text{Green mould inhibition (\%)} = (C - T) \times 100/(C - 6)$$

where: C – diameter of the pathogen colony of the control group; 6 – diameter of the pathogen disk; T – diameter of the pathogen colony after treatments

Inhibitory effect of bacterial culture on the fungal growth and spore germination and production. The inhibitory effect of the bacterial culture on spore ger-

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mination, fungal growth and spore production of the pathogenic fungus was observed using the following method with some minor modifications (Thonglem *et al.* 2007). An equal volume of *P. digitatum* spore suspension (3×10^6 CFU/ml) was mixed with the bacterial culture in sterile test tubes containing 5.0 ml of Potato Dextrose Broth (PDB; Difco). The control was treated only with *P. digitatum* spore suspension under the same conditions. Then they were incubated on a rotary shaker at 120 rpm at 25°C. The mycelial growth, spore germination, and spore production of the pathogenic fungus were investigated after 12 h incubation. In addition, another set of the experiment was prepared by incubating the mixture of spore suspension and bacterial culture (1 : 1, v/v) at room temperature for 7 days, and spore germination was observed. Spore germination, mycelial growth, and spore production of the fungus were determined using light microscopy.

Antagonism test using lemon fruits. The lemon fruits were selected free of wounds and rots, previously untreated with fungicide, and as homogenous as possible in maturity and size, and were stored at 5°C under dry conditions until use. Afterwards, they were surface disinfected by immersion in a dilute solution of ethanol (70%) for 1 min, washed twice by immersion in distilled and sterilised distilled water (sdH_20), and left in a dry place to remove excess water on the surface until used for *in vivo* assays (Kotan *et al.* 2009). According to the results of the antifungal activity assays *in vitro* and inhibitory effect of the bacterial culture on the mycelial growth, spore production, and spore germination of the pathogenic fungus, two bacteria including TV-17C and A-16 isolates were selected for *in vivo* assays. Each lemon fruit was dipped into the bacterial suspensions 1×10^8 CFU/ml. The pathogen application was conducted at four different times including the application of bacteria and pathogenic fungi at the same time, the application of pathogenic fungi after 24, 48, and 72 h from the application of the bacterial suspension incubated under storage conditions. The pathogen was prepared from approximately 10-day-old cultures grown on PDA medium. The spore suspension containing 1×10^5 CFU/ml prepared Glucose Yeast Broth (BYB) was supplemented with 1 ml of Tween 20 (Pimenta *et al.* 2008). The pathogen application was conducted at four different times including simultaneously with the application of bacteria and pathogenic fungi, and the application of pathogenic fungi after 24, 48, and 72 h from the application of bacterial suspension incubated under storage conditions.

Statistical analyses. All data in the present study were processed by JUMP 5.0 and the means were separated by LSMeans Student's tests. The statistical analyses of percentage values in relation to the fruit set were performed using transformed values.

RESULTS AND DISCUSSION

The list of the tested bacterial isolates and pathogenic fungus is presented in Table 1. Ten bacterial isolates consisted of 5 different species and 2 genera according to both MIS and BIOLOG were tested in this study. Similarity index was changed varied from 0.494 to

Table 1. Bio-control bacterial isolates and pathogenic fungus, their identification results, and similarity indexes (SIM) according to Microbial Identification (MIS) and BIOLOG systems, hypersensitivity (HR), and pathogenicity (PAT) test results

Isolates	MIS results	SIM	Biolog results	SIM	Isolated from	HR	PAT	Reference
TV-6F	*Bacillus subtilis*	0.831	*Bacillus subtilis*	0.56	Graminea	–	–	Çakmakci *et al.* (2010)
TV-12H	*Bacillus subtilis*	0.744	*Bacillus subtilis*	0.53	Graminea	–	–	Çakmakci *et al.* (2010)
TV-17C	*Bacillus subtilis*	0.677	*Bacillus subtilis*	0.76	raspberry			Çakmakci *et al.* (2010)
TV-73 F	*Bacillus pumilus*	0.594	*Bacillus pumilus*	0.54	sedum	–	–	Çakmakci *et al.* (2010)
TV-79 B	*Bacillus cereus*	0.494	*Bacillus cereus*	0.48	sedum	–	–	Çakmakci *et al.* (2010)
TV-103B	*Bacillus megaterium*	0.514	*Bacillus megaterium*	0.45	Graminea	–	–	Çakmakci *et al.* (2010)
RK-103	*Bacillus pumilus*	0.626	*Bacillus pumilus*	0.47	apple	–	–	Kotan *et al.* (2009)
A-16	*Agrobacterium radiobacter*	0.786	*Agrobacterium rubi*	0.56	apple	–	–	in this study
EK-7	*Bacillus subtilis*	0.624	*Bacillus subtilis*	0.74	rosehip	–	–	Tozlu *et al.* (2016)
BA-140	*Bacillus subtilis*	0.598	*Bacillus subtilis*	0.43	soil	–	–	Kotan (1998)
PD-1	*Penicillium digitatum*	0.786	*Penicillium digitatum*	0.67	lemon	NT	+	in this study

+ positive reaction; – negative reaction; NT – not tested

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Table 2. Enzyme activities of the bacterial isolates

Bacterial isolates	Enzyme activities of bacteria		
	chitinase (U/ml)	glucanase (mm)	protease (mm)
TV-6F	65.0	26	30
TV-12H	74.0	24	34
TV-17C	67.0	30	41
TV-73 F	60.0	27	35
TV-79 B	60.5	27	33
TV-103B	58.5	29	40
RK-103	65.0	25	32
A-16	85.0	32	00
EK-7	66.5	28	37
BA-140	65.5	28	33
Control (*P. digitatum*)	NT	NT	NT

NT – not tested

0.831 for MIS and from 0.43 to 0.76 for Biolog. According to MIS and BIOLOG results, TV-6F, TV-12H, TV-17C, EK-7, BA-140 were defined as *Bacillus subtilis*, TV-73F, RK-103 as *B. pumilus*, TV-79B as *B. cereus* and TV-103B as *B. megaterium*. A-16 was defined as *Agrobacterium radiobacter* according to MIS and *A. rubi* according to BIOLOG results. In our previous study, these bacterial isolates were also used for the biological control of *P. digitatum* (Mohammadi *et al.* 2014).

Hypersensitivity and pathogenicity tests results of the bacteria used in this research were negative. However, the fungus tested was highly virulent on lemon fruits (Figure 1).

The results of enzymes activities were presented in Table 2 and antifungal assays were presented in Table 4 and Figure 2. Chitinase enzyme activities ranged from 58.5 (TV-103 B) to 85.0 U/ml (A-16) and those of glucanase from 24 (TV-12H) to 32 mm (A-16) and protease from 00 (A-16) to 41 mm (TV-17C). All tested isolates produced chitinase, glucanase, and protease enzyme inhibited fungal growth and reduced the growth rate of the pathogen.

Figure 1. Pathogenicity test results of *Penicillium digitatum* on lemon fruits treated with only the pathogenic fungus (**A**) and only sterile water (**B**)

The degradation of fungal cell walls with the production of hydrolytic enzymes of bacterial isolates is one of the most important mechanisms for bio-control of phytopathogenic fungi (Weller 2007; Elshafie *et al.* 2012). These enzymes such as chitinase (Ordentlich *et al.* 1988), protease (Saligkarias *et al.* 2002), and glucanase (Leelasuphakul *et al.* 2006). Senol *et al.* (2014) reported that the volatile metabolic assay suggested that metabolic substances and cell wall degrading enzymes may contribute to the inhibition of fungal growth and causing ultrastructure defects in fungal hypha and spores.

Inhibitory effects of the bacterial culture on the spore germination, fungal growth, and spore production of the pathogenic fungus are presented in Table 3. A-16 and TV-17C inhibited spore germination and spore production (Table 3 and Figure 3). For this reason, they were selected for *in vivo* assays on lemon fruits.

P. digitatum survives in the orchard from season to season mainly in the form of conidia and causes infection by airborne spores where there are injuries or blemishes (Chang & Petersen 2003). It was reported that reported that spore production and germination of pathogenic green mold were inhibited by *B. subtilis* and *A. radiobacter* (Ketabchi *et al.* 2012). Green mould sporulation continues during storage and transportation, and the protecting applications are a valuable strategy against the pathogen infection takings place after harvest under storage conditions (Guo *et al.* 2014). Therefore, the inhibition of spore production of *P. digitatum* by A-16 and TV-17C was very important and would have great advantage for suppression of the post-harvest disease.

Table 3. Inhibitory effect of the bacterial culture of TV- 17C and A-16 isolates on the mycelial growth, spore production and spore germination of the pathogenic fungus

Isolates	Bacterial species	Mycelial growth	Spore	
			production	germination
TV-17C	*B. subtilis*	+	–	–
A-16	*A. radiobacter*	+	–	–
Control		+	+	+

+ spore germination (mycelial growth and spore production of the fungus were observed); – not observed

doi: 10.17221/55/2016-PPS

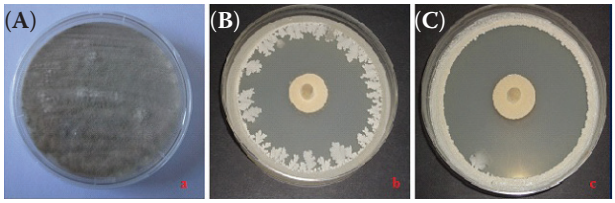

Figure 2. Antifungal activity assays on Petri plates: (**A**) Control (only pathogen), (B) *B. subtilis*, (**C**) *A. radiobacter*

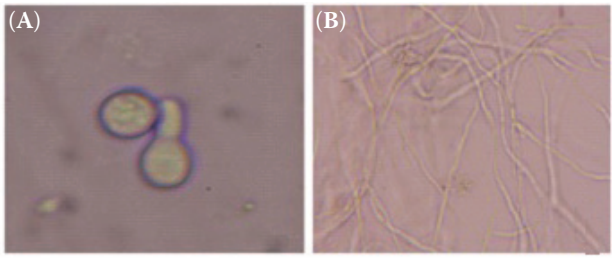

Figure 3. Spore germination in control application (**A**) and *A. radiobacter* application (**B**)

The postharvest disease control relies on the use of synthetic fungicides (Ecker & Ogawa 1988) such as imazalil, sodium *ortho*-phenylphenate or thiabendazole (Yildiz *et al*. 2005; Torres *et al*. 2007). However, these chemical agents have been applied for many years with few little or limited success due to the development of resistance by the fungus (Zamani *et al.* 2006). In addition, the accumulation of hazardous chemicals in the environment raises public concern about their effect on human health and generates environmental concerns mainly due to the carcinogenic and/or teratogenic properties of the compounds as well as their cumulative toxic effects (Thonglem *et al*. 2007; Pimenta *et al*. 2008). In this respect, microbial bio-control agents have shown a great potential as an alternative to synthetic fungicides and offer an environmentally friendly alternative to the use of synthetic pesticides (Kotan *et al*. 2009).

There are many studies demonstrating the postharvest disease control by using biological agents (Zamani *et al*. 2006; Thonglem *et al*. 2007; Torres *et al*. 2007; Leelasuphakul *et al*. 2008; Pimenta *et al*. 2008; Kotan *et al*. 2009; Sharma *et al*. 2009; Panebianco *et al*. 2015).

According to the results of antifungal assays in this study, all bacterial isolates showed more or less antifungal activity against the pathogen compared to the control *in vitro*. The mycelial growth of the pathogenic fungus ranged from 74.47% to 84.04% in dual culture, from 65.49% to 80.76% in to using cell-free bacterial culture and from 58.33% to 79.00% in volatile metabolites (Table 4). The most promising results were obtained from A-16 and TV-17C in dual culture, cell-free bacterial culture, and volatile metabolites (Table 4 and Figure 2).

The results of *in vivo* assays are presented in Table 5. Both A-16 and TV-17C significantly reduced disease severity on lemon fruits. The pathogenic fungi were applied after 24, 48, and 72 h from the application of bacterial suspension. While the largest lesion diameter was observed in the application of bacteria and

Table 4. Antifungal activities of whole culture, cell-free bacterial culture, and volatile metabolites of tested bacteria

Applications	Mean inhibition of fungi (%)		
	dual culture	cell-free filtrates	volatile metabolites
A-16	84.04^{A}	80.76^{A}	79.00^{A}
TV-17C	82.40^{AB}	79.84^{A}	77.33^{AB}
TV-73F	80.46^{BC}	76.25^{BC}	73.85^{C}
EK-7	80.37^{BC}	77.31^{B}	75.97^{B}
BA-140	79.20^{CD}	76.15^{BC}	72.94^{C}
TV-79B	78.63^{CD}	72.51^{D}	70.09^{D}
TV-12H	77.98^{CD}	74.78^{C}	71.20^{D}
RK-103	77.54^{CDE}	65.49^{E}	58.33^{F}
TV-6F	76.76^{DE}	65.74^{E}	59.19^{F}
TV-103B	74.47^{E}	66.65^{E}	61.68^{E}
Control	2.68^{F}	0.56^{F}	0.09^{G}
LSD	3.13	1.85	1.71
CV	2.55	3.23	1.58

Values followed by different letters are significantly different at $P < 0.01$

Table 5. The effect of the cell suspension of bio-control bacteria applied at four different times on lesion diameter on lemon fruits compared with the control

Aplications	Lesion diameters of decay on fruits (cm)			
	simultaneously	after 24 h	after 48 h	after 72 h
TV-17C	7.16^{A}	5.63^{B}	4.71^{B}	2.09^{B}
A-16	7.96^{B}	5.2^{A}	4.13^{A}	1.49^{A}
Control	7.95^{B}	7.83^{C}	7.85^{C}	7.71^{C}
CV	1.60	3.67	1.50	3.15
LSD	1.79	0.33	0.121	0.17
$CV_{treatment}$	32.09			
LSD	1.36			
$CV_{bacterial\ isolates}$	31.42			
LSD	1.15			

Values followed by different letters are significantly different at $P < 0.01$

doi: 10.17221/55/2016-PPS

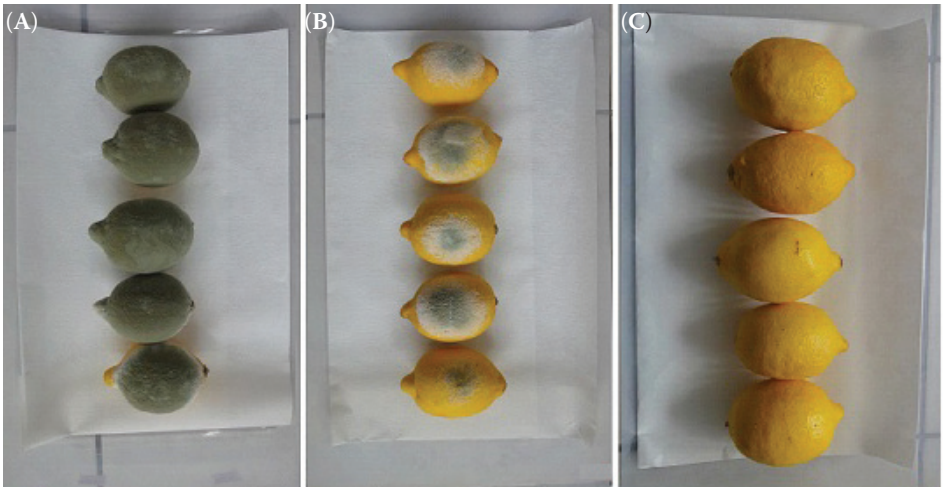

Figure 4. Lemon fruits treated with only *P. digitatum* (**A**), together the cell suspension of antagonistic bacteria *B. subtilis* and *P. digitatum* (**B**), and negative control (after 72 h) (**C**)

pathogenic fungi at the same time, the smallest lesion diameter was observed when the pathogen have applied after 72 h from the application of bacterial suspension. Previous reports showed that induced resistance for pathogenic fungi was positively correlated with increased incubation time after the application of the antagonists (Nantawanit *et al.* 2010; Lu *et al.* 2013). It was reported that the sufficient bacterial population on the fruit surface are very important for the effectiveness of bio-control bacteria (Benbow & Sugar 1999). Similarly, this study showed that the percentage of infection and disease severity significantly decreased with the extension of the incubation time of bio-control bacteria on lemon fruits.

A-16 and TV-17C applications were very effective on disease suppression and lesion diameters, and 1.49 and 2.09 cm lesion diameters on lemon fruits were observed after 72 h, respectively. Lemon fruits treated together the cell suspension of antagonistic bacteria *B. subtilis* and *P. digitatum* were in Figure 4. As the research shows, A-16 application was the most effective against *P. digitatum* on lemon fruits. Furthermore, the cell suspension of A-16 completely reduced disease severity in comparison with the positive control on lemon fruits under storage conditions for 45 days (Figure 5).

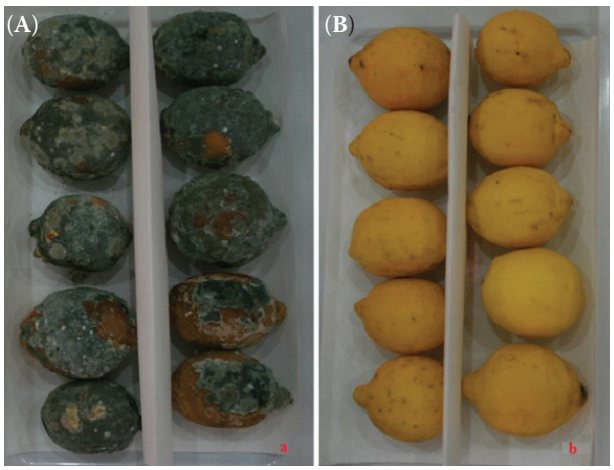

Figure 5. Lemon fruits treated with only *P. digitatum* (**A**), the cell suspension of *A. radiobacter* and *P. digitatum* applied in 72 h from the application of the bacteria stored for 45 days (**B**)

The *in vitro* and *in vivo* data support that antagonism is the main mechanism of *A. radiobacter* for the biological control of the disease, but the antimicrobial compounds have not been isolated and identified yet. *A. radiobacter* has been recognised as an opportunistic human pathogen responsible for nosocomial infections, they are mainly bacteraemia, peritonitis, and urinary tract infections (Edmond *et al.* 1993). Besides these highly specialised bacteria, non-phytopathogenic isolates were also found in diverse environments not always in association with plants. However, there has not been a study so far showing that *A. radiobacter* is used as a bio-control agent of post-harvest diseases. The first bacterium called *A. radiobacter* isolate K 84 (currently isolate K-1026) and produced Agrocin 84 antibiotic were registered in the United States Environmental Protection agency (EPA) for the control of crown gall in 1979 (Kerr 1980). To our knowledge, this is the first report of *A. radiobacter* (A-16) being used for the biological control of post-harvest plant diseases.

B. subtilis was the other bio-control agent of this research. There are many studies showing that *Bacillus* occurring naturally on the surface of fruits or vegetables is used as a bio-control agent of post-harvest diseases (Kotan *et al.* 2009; Elshafei *et al.* 2012; Ketabchi *et al.* 2012; Mohammadi *et al.*

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2014). *Bacillus* species, particularly *B. subtilis*, *B. cereus*, and *B. amyloliquefaciens* have been successfully employed in pest and disease management programs (Nagorska *et al.* 2007; Ji *et al.* 2008; Francis *et al.* 2010). The bacteria of the genus *Bacillus* have a a great potential as a biological control agent because they keep their viability with long-term storage (Nagorska *et al.* 2007; Ongena & Jacques 2008). Considering the cases mentioned above, the use of Gram-positive bacteria having a natural formulation in this disease suppression would have a great advantage and a contribution to overcoming the drawbacks incurred by the use of other bio-control agents (Kotan *et al.* 2009). *B. subtilis* produces antibiotics and various biologically active compounds with a broad spectrum of activities against plant pathogens that are able to induce systemic resistance (Stein 2005; Nagorska *et al.* 2007; Ongena & Jacques 2008). Moreover, *B. subtilis* cells can produce dormant spores that are resistant to extreme conditions and thus can be easily formulated and stored (Piggot & Hilbert 2004).

CONCLUSION

The present study showed that *B. subtilis* and *A. radiobacter* may be useful as potential bio-control agents against *P. digitatum*. These isolates, especially *B. subtilis,* can be used as bio-control agents for the post-harvest decay of citrus. Hence, further study is necessary to develop a long-term carrier material, to complete cytotoxicity using human cell, ecotoxicity, and toxicity tests of these bacterial isolates, specifically *A. radiobacter*, on target organisms.

Acknowledgement. The authors would like to thank Atatürk University English Editorial Services for the revision of the manuscript. We have published the present research results as an abstract of the Proceedings of the 21st Iranian Plant Protection Congress, Urmia, Iran.

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Received: 2016–04–04
Accepted after corrections: 2017–02–23
Published online: 2017–04– 07