

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

PARISA MOHAMMADI<sup>1</sup>, ELIF TOZLU<sup>1\*</sup>, RECEP KOTAN<sup>1</sup> and MERVE ŞENOL KOTAN<sup>2</sup>

<sup>1</sup>Department of Plant Protection and <sup>2</sup>Department of Agricultural Biotechnology, Faculty of Agriculture, Ataturk University, Erzurum, Turkey

\*Corresponding author: elifalpertzlu@atauni.edu.tr

### 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

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 brasiliensis*, *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 bio-control 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; ÇAKMAKÇI *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 ( $1 \times 10^8$  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<sub>2</sub>O) 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 ( $1 \times 10^8$  CFU/ml) and spore suspension ( $1 \times 10^5$  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 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  $1 \times 10^8$  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-

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 ( $3 \times 10^6$  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<sub>2</sub>O), 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  $1 \times 10^8$  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  $1 \times 10^5$  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	<i>Bacillus subtilis</i>	0.831	<i>Bacillus subtilis</i>	0.56	Graminea	–	–	ÇAKMAKCI <i>et al.</i> (2010)
TV-12H	<i>Bacillus subtilis</i>	0.744	<i>Bacillus subtilis</i>	0.53	Graminea	–	–	ÇAKMAKCI <i>et al.</i> (2010)
TV-17C	<i>Bacillus subtilis</i>	0.677	<i>Bacillus subtilis</i>	0.76	raspberry			ÇAKMAKCI <i>et al.</i> (2010)
TV-73 F	<i>Bacillus pumilus</i>	0.594	<i>Bacillus pumilus</i>	0.54	sedum	–	–	ÇAKMAKCI <i>et al.</i> (2010)
TV-79 B	<i>Bacillus cereus</i>	0.494	<i>Bacillus cereus</i>	0.48	sedum	–	–	ÇAKMAKCI <i>et al.</i> (2010)
TV-103B	<i>Bacillus megaterium</i>	0.514	<i>Bacillus megaterium</i>	0.45	Graminea	–	–	ÇAKMAKCI <i>et al.</i> (2010)
RK-103	<i>Bacillus pumilus</i>	0.626	<i>Bacillus pumilus</i>	0.47	apple	–	–	KOTAN <i>et al.</i> (2009)
A-16	<i>Agrobacterium radiobacter</i>	0.786	<i>Agrobacterium rubi</i>	0.56	apple	–	–	in this study
EK-7	<i>Bacillus subtilis</i>	0.624	<i>Bacillus subtilis</i>	0.74	rosehip	–	–	TOZLU <i>et al.</i> (2016)
BA-140	<i>Bacillus subtilis</i>	0.598	<i>Bacillus subtilis</i>	0.43	soil	–	–	KOTAN (1998)
PD-1	<i>Penicillium digitatum</i>	0.786	<i>Penicillium digitatum</i>	0.67	lemon	NT	+	in this study

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

doi: 10.17221/55/2016-PPS

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 ( <i>P. digitatum</i> )	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-73E, 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 activi-



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

ties 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.

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	<i>B. subtilis</i>	+	–	–
A-16	<i>A. radiobacter</i>	+	–	–
Control		+	+	+

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

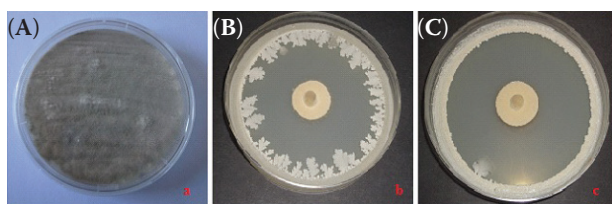


Figure 2. Antifungal activity assays on Petri plates: (A) Control (only pathogen), (B) *B. subtilis*, (C) *A. radiobacter*

The postharvest disease control relies on the use of synthetic fungicides (ECKER & OGAWA 1988) such as imazalil, sodium *ortho*-phenylphenate or thia-bendazole (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 post-harvest disease control by using biological agents

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 <sup>A</sup>	80.76 <sup>A</sup>	79.00 <sup>A</sup>
TV-17C	82.40 <sup>AB</sup>	79.84 <sup>A</sup>	77.33 <sup>AB</sup>
TV-73F	80.46 <sup>BC</sup>	76.25 <sup>BC</sup>	73.85 <sup>C</sup>
EK-7	80.37 <sup>BC</sup>	77.31 <sup>B</sup>	75.97 <sup>B</sup>
BA-140	79.20 <sup>CD</sup>	76.15 <sup>BC</sup>	72.94 <sup>C</sup>
TV-79B	78.63 <sup>CD</sup>	72.51 <sup>D</sup>	70.09 <sup>D</sup>
TV-12H	77.98 <sup>CD</sup>	74.78 <sup>C</sup>	71.20 <sup>D</sup>
RK-103	77.54 <sup>CDE</sup>	65.49 <sup>E</sup>	58.33 <sup>F</sup>
TV-6F	76.76 <sup>DE</sup>	65.74 <sup>E</sup>	59.19 <sup>F</sup>
TV-103B	74.47 <sup>E</sup>	66.65 <sup>E</sup>	61.68 <sup>E</sup>
Control	2.68 <sup>F</sup>	0.56 <sup>F</sup>	0.09 <sup>G</sup>
LSD	3.13	1.85	1.71
CV	2.55	3.23	1.58

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

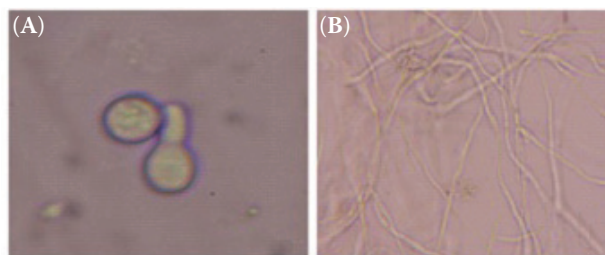


Figure 3. Spore germination in control application (A) and *A. radiobacter* application (B)

(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 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

Applications	Lesion diameters of decay on fruits (cm)			
	simulta-neously	after 24 h	after 48 h	after 72 h
TV-17C	7.16 <sup>A</sup>	5.63 <sup>B</sup>	4.71 <sup>B</sup>	2.09 <sup>B</sup>
A-16	7.96 <sup>B</sup>	5.2 <sup>A</sup>	4.13 <sup>A</sup>	1.49 <sup>A</sup>
Control	7.95 <sup>B</sup>	7.83 <sup>C</sup>	7.85 <sup>C</sup>	7.71 <sup>C</sup>
CV	1.60	3.67	1.50	3.15
LSD	1.79	0.33	0.121	0.17
CV <sub>treatment</sub>			32.09	
LSD			1.36	
CV <sub>bacterial isolates</sub>			31.42	
LSD			1.15	

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

doi: 10.17221/55/2016-PPS

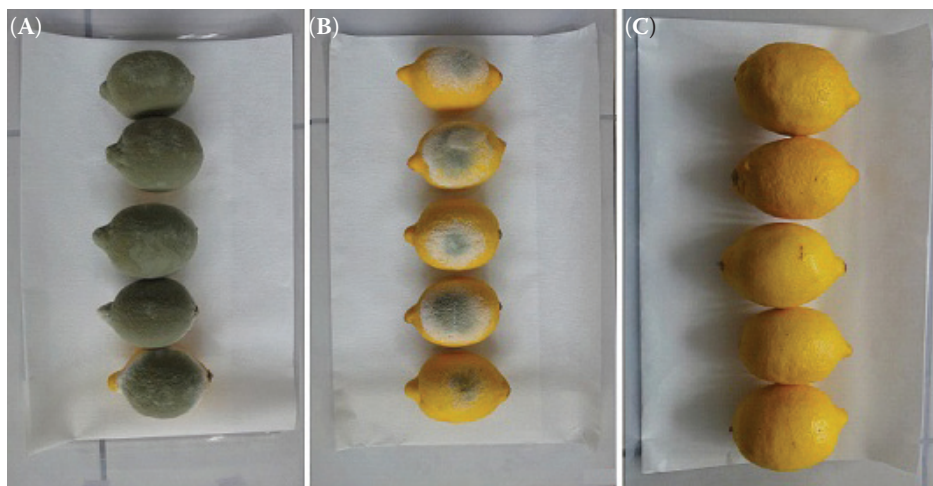


Figure 4. Lemon fruits treated with only *P. digitatum* (A), together the cell suspension of antagonistic bacteria *B. subtilis* and *P. digitatum* (B), and negative control (after 72 h) (C)

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).

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.*

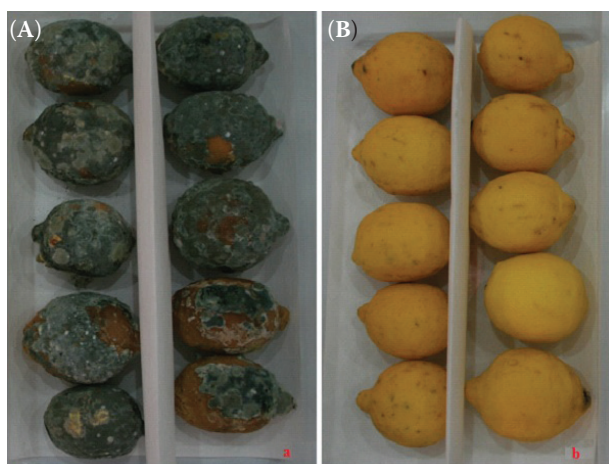


Figure 5. Lemon fruits treated with only *P. digitatum* (A), the cell suspension of *A. radiobacter* and *P. digitatum* applied in 72 h from the application of the bacteria stored for 45 days (B)

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 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 21<sup>st</sup> Iranian Plant Protection Congress, Urmia, Iran.

## References

- Atlas R.M. (1997): Handbook of Microbiological Media. Boca Raton, CRC Press.
- Benbow J.M., Sugar D. (1999): Fruit surface colonization and biological control of postharvest diseases of pear by pre-harvest yeast applications. *Plant Disease*, 83: 839–844.
- Çakmakçı R., Erman M., Kotan R., Çığ F., Karagöz K., Sezen M. (2010): Growth promotion and yield enhancement of sugar beet and wheat by application of plant growth promotion rhizobacteria. In: Proceedings International Conference on Organic Agriculture in Scope of Environmental Problems, Feb 3–7, 2010, Famagusta, Cyprus Island: 198–202.
- Chang W.N., Petersen J.B. (2003): *Citrus Production: a Manual for Asian Farmers*. Taipei, Food and Fertilizer Technology Center for the Asian and Pacific Region. Available at <https://www.cabdirect.org/cabdirect/abstract> (accessed Apr 1, 2016).
- D'Aquino S., Schirra M., Palma A., Angioni A., Cabras P., Migheli Q. (2006): Residue levels and effectiveness of pyrimethanil vs imazalil when using heated postharvest dip treatments for control of *Penicillium* decay on citrus fruit. *Journal of Agricultural and Food Chemistry*, 54: 4721–4726.
- Eckert J.W., Ogawa J.M. (1988): The chemical control of post-harvest diseases: deciduous fruit, berries, vegetables and root/tuber crops. *Annual Review of Phytopathology*, 26: 433–469.
- Eckert J.W., Eaks I.L. (1989): Postharvest disorders and diseases of citrus fruits. In: Reuter W., Calavan E.C., Carman G.E. (eds): *The Citrus Industry*. Berkeley, University of California Press: 179–260.
- Edmond M.B., Riddler S.A., Baxter C.M., Wicklund B.M., Pascuile A.W. (1993): *Agrobacterium radiobacter* a recently recognized opportunistic pathogen. *Clinical Infectious Disease*, 16: 388–391.
- Elshafie H.S., Camele I., Racioppi R., Scranò L., Iacobellis N.S., Bufo S.A. (2012): *In vitro* antifungal activity of *Burkholderia gladioli* pv. *agaricicola* against some phytopathogenic fungi. *International Journal of Molecular Science*, 13: 16291–16302.
- Evmert M.K., Gabriele B.G., Piechulla B. (2007): Volatiles of bacterial antagonists inhibit mycelial growth of the plant pathogen *Rhizoctonia solani*. *Archives of Microbiology*, 187: 351–360.
- FAO (2015): Value of agricultural production date. Available at <http://faostat3.fao.org/home/E> (accessed Apr 3, 2016).
- Francis I., Holsters M., Vereecke D. (2010): The Gram-positive side of plant-microbe interactions. *Environmental Microbiology*, 12: 1–12.
- Guo J., Fang W., Lu H., Zhu R., Lu L., Zheng X., Yu T. (2014): Inhibition of green mold disease in mandarins by preventive applications of methyl jasmonate and antagonistic yeast *Cryptococcus laurentii*. *Postharvest Biology and Technology*, 88: 72–78.
- Holmes G.J., Eckert J.W. (1999): Sensitivity of *Penicillium digitatum* and *P. italicum* to postharvest citrus fungicides in California. *Phytopathology*, 89: 716–721.
- Holmes B., Costas M., Ganner M., On S.L., Stevens M. (1994): Evaluation of Biolog system for identification of some gram negative bacteria of clinical importance. *Journal of Clinical Microbiology*, 32: 1970–1975.



doi: 10.17221/55/2016-PPS

- Ismail M., Zhang J. (2004): Post-harvest citrus diseases and their control. *Outlooks on Pest Management*, 15: 29–35.
- Ji X.L., Lu G.B., Gai Y.P., Zheng C.C., Mu Z.M. (2008): Biological control against bacterial wilt and colonization of mulberry by an endophytic *Bacillus subtilis* strain. *FEMS Microbiology Ecology*, 65: 565–573.
- Kanetis L., Förster H., Adaskaveg J.E. (2007): Comparative efficacy of the new postharvest fungicides azoxystrobin, fludioxonil, and pyrimethanil for managing citrus green mold. *Plant Disease*, 91: 1502–1511.
- Kerr A. (1980): Biological control of crown gall through production of Agrocin 84. *Plant Disease*, 64: 25–30.
- Ketabchi S., Taghipour M.A., Sharzei A. (2012): Identification of lime fruit surface colonizing bacteria antagonistic against the green mold and comparison of biological control with heat treatment and chemical control. *Asian Journal of Experimental Biological Sciences*, 3: 287–292.
- Klement Z., Farkas G.L., Lovrekovich L. (1964): Hypersensitive reaction induced by phytopathogenic bacteria in the tobacco leaf. *Phytopathology*, 54: 474–477.
- Kotan R. (1998): Biological and chemical control of bacterial leaf spot (*Xanthomonas campestris* pv. *vesicatoria* (Doidge) Dye.) on pepper and tomato. [Master Thesis.] Atatürk University, Turkey.
- Kotan R., Dikbaş N., Bostan H. (2009): Biological control of postharvest disease caused by *Aspergillus flavus* on stored lemon fruits. *African Journal of Biotechnology*, 8: 209–214.
- Leelasuphakul W., Sivanunsakul P., Phongpaichit S. (2006): Purification, characterization and synergistic activity of  $\beta$ -1,3-glucanase and antibiotic extract from an antagonistic *Bacillus subtilis* NSRS 89-24 against rice blast and sheath blight pathogens. *Enzyme and Microbial Technology*, 38: 990–997.
- Leelasuphakul W., Hemmanee P., Chuenchitt S. (2008): Growth inhibitory properties of *Bacillus subtilis* strains and their metabolites against the green mold pathogen (*Penicillium digitatum* Sacc.) of citrus fruit. *Postharvest Biology and Technology*, 48: 113–121.
- Lu L., Lu H., Wu C., Fang W., Yu C., Ye C., Shi Y., Yu T., Zheng X. (2013): *Rhodosporidium paludigenum* induces resistance and defense-related responses against *Penicillium digitatum* in citrus fruit. *Postharvest Biology and Technology*, 85: 196–202.
- Macarasin D., Cohen L., Eick A., Rafael G., Belausov E., Wisniewski M., Droby S. (2007): *Penicillium digitatum* suppresses production of hydrogen peroxide in host tissue during infection of citrus fruit. *Phytopathology*, 97: 1491–1500.
- Maldonado M.C., Corona J., Gordillo M.A., Navarro A.R. (2009): Isolation and partial characterization of antifungal metabolites produced by *Bacillus* sp. IBA 33. *Current Microbiology*, 59: 646–650.
- Mari M., Iori R., Leoni O., Marchi A. (1993): *In vitro* activity of glucosinolate-derived isothiocyanates against postharvest fruit pathogens. *Annals of Applied Biology*, 123: 155–164.
- Mehmood Z., Ahmed I., Mohammad F., Ahmed S. (1999): Indian medicinal plants: a potential source of anticancer drug. *Pharmaceutical Biology*, 37: 237–242.
- Miller G.L. (1959): Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical Chemistry*, 31: 426–428.
- Miller L.T. (1982): Single derivatization method for routine analysis of bacterial whole-cell fatty acid methyl esters, including hydroxy acids. *Journal of Clinical Microbiology*, 16: 584–586.
- Mohammadi P., Tozlu E., Kotan R. (2014): Screening of antagonistic bacteria for biological control of green mold caused by *Penicillium digitatum* Sacc. of citrus fruits. In: *Proceeding 21<sup>th</sup> Iranian Plant Protection Congress*, Jan 21–22, 2014, Urmia, Iran: 189.
- Moss M.O. (2008): Fungi quality and safety issues in fresh fruits and vegetables. *Journal of Applied Microbiology*, 104: 1239–1243.
- Nagorska K., Bikowski M., Obuchowski M. (2007): Multicellular behaviour and production of a wide variety of toxic substances support usage of *Bacillus subtilis* as a powerful biocontrol agent. *Acta Biochimica Polonica*, 54: 495–508.
- Nantawanit N., Chanchaichaovivat A., Panijpan B., Ruenwongsa P. (2010): Induction of defense response against *Colletotrichum capsici* in chili fruit by the yeast *Pichia guilliermondii* strain R13. *Biological Control*, 52: 145–152.
- Ongena M., Jacques P. (2008): *Bacillus* lipopeptides: versatile weapons for plant disease biocontrol. *Trends in Microbiology*, 16: 115–125.
- Ordentlich A., Elad Y., Chet I. (1988): The role of chitinase of *Serratia marcescens* in biocontrol *Sclerotium rolfsii*. *Phytopathology*, 78: 84–87.
- Panebianco S., Vitale A., Polizzi G., Scala F., Cirvilleri G. (2015): Enhanced control of postharvest citrus fruit decay by means of the combined use of compatible biocontrol agents. *Biological Control*, 84: 19–27.
- Piggot P.J., Hilbert D.W. (2004): Sporulation of *Bacillus subtilis*. *Current Opinion of Microbiology*, 7: 579–586.
- Pimenta R.S., Silva F.L., Silva J.F.M., Morais P.B., Braga D.T., Rosa C.A., Corrêa A. (2008): Biological control of *Penicillium italicum*, *P. digitatum* and *P. expansum* by the predacious yeast *Saccharomycopsis schoenii* on oranges. *Brazilian Journal of Microbiology*, 39: 85–90.
- Saligkari I.D., Gravanis E.T., Harry A.S. (2002): Biological control of *Botrytis cinerea* on tomato plants by the use of

- epiphytic yeasts *Candida guilliermondii* strains 101 and US 7 and *Candida oleophila* strain I-182: II. A study on mode of action. *Biological Control*, 25: 151–161.
- Sasser M. (1990): Identification of bacteria through fatty acid analysis. In: Klement Z., Rudolph K., Sands D. (eds): *Methods in Phytobacteriology*. Budapest, Academia Kiado: 199–204.
- Schirra M., D'Aquino S., Palma A., Marceddu S., Angioni A., Cabras P., Scherm B., Migheli Q. (2005): Residue level, persistence and storage performance of citrus fruit treated with fludioxonil. *Journal of Agricultural and Food Chemistry*, 53: 6718–6724.
- Schirra M., Palma A., Barberis A., Angioni A., Garau V.L., Cabras P., D'Aquino S. (2010): Postinfection activity, residue levels, and persistence of azoxystrobin, fludioxonil, and pyrimethanil applied alone or in combination with heat and imazalil for green mold control on inoculated oranges. *Journal of Agricultural and Food Chemistry*, 58: 3661–3666.
- Senol M., Nadaroglu H., Dikbas N., Kotan R. (2014): Purification of Chitinase enzymes from *Bacillus subtilis* bacteria TV-125, investigation of kinetic properties and antifungal activity against *Fusarium culmorum*. *Annals of Clinical Microbiology and Antimicrobials*, 13, 35. doi: 10.1186/s12941-014-0035-3
- Sharma R.R., Singh D., Singh R. (2009): Biological control of postharvest diseases of fruits and vegetables by microbial antagonists: a review. *Biological Control*, 50: 205–221.
- Smilanick J.L., Mansour M.F., Margosan D.A., Gabler F.M., Goodwine W.R. (2005): Influence of pH and NaHCO<sub>3</sub> on effectiveness of imazalil to inhibit germination of *Penicillium digitatum* and to control postharvest green mold on citrus fruit. *Plant Disease*, 89: 640–648.
- Smilanick J.L., Mansour M.F., Mlikota-Gabler F., Goodwine W.R. (2006): The effectiveness of pyrimethanil to inhibit germination of *Penicillium digitatum* and to control citrus green mold after harvest. *Postharvest Biology and Technology*, 42: 75–85.
- Stein T. (2005): *Bacillus subtilis* antibiotics: structures, syntheses and specific functions. *Molecular Microbiology* 56: 845–857.
- Talibi I., Boubaker H., Boudyach E.H., Ait Ben Aoumar A. (2014): Alternative methods for the control of postharvest citrus diseases. *Journal of Applied Microbiology*, 117: 1–17.
- Teather R.M., Wood P.J. (1998): Use of Congo red-polysaccharide interaction in enumeration and characterization of cellulolytic bacteria from the bovine rumen. *Applied and Environmental Microbiology*, 43: 777–780.
- Thonglem K., Plikomol A., Pathom-aree W. (2007): Growth inhibition of *Penicillium digitatum* by antagonistic microorganisms isolated from various parts of orange tree. *Maejo International Journal of Science and Technology*, 1: 208–215.
- Tozlu E., Mohammadi P., Senol Kotan M., Nadaroglu H., Kotan R. (2016): Biological control of *Sclerotinia sclerotiorum* (Lib.) de Bary, the causal agent of white mould disease in red cabbage, by some bacteria. *Plant Protection Science*, 52: 188–198
- Torres R., Nunes C., Garcia J.S., Abadias M., Vinas I., Manso T., Olmo M., Usall J. (2007): Application of *Pantoea agglomerans* CPA-2 in combination with heated sodium bicarbonate solutions to control the major postharvest diseases affecting citrus fruit at several mediterranean locations. *European Journal of Plant Pathology*, 118: 73–83.
- Weller D.M. (2007): *Pseudomonas* biocontrol agents of soilborne pathogens: Looking back over 30 years. *Phytopathology*, 97: 250–256.
- Yildiz F., Kinay P., Yildiz M., Sen F., Karacali I. (2005): Effects of preharvest applications of CaCl<sub>2</sub>, 2,4-D and benomyl and postharvest hot water, yeast and fungicide treatments on development of decay on *Satsuma mandarins*. *Journal of Phytopathology*, 153: 94–98.
- Zamani M., Tehrani A.S., Ahmadzadeh M., Abadi A.A. (2006): Effect of fluorescent pseudomonades and *Trichoderma* sp. and their combination with two chemicals on *Penicillium digitatum* caused agent of citrus green mold. *Communications in Agricultural and Applied Biological Sciences*, 71: 1301–1310.
- Zamani M., Tehrani A.S., Ahmadzadeh M., Hosseininaveh V., Mostofy Y. (2009): Control of *Penicillium digitatum* on orange fruit combing *Pantoea agglomerans* with hot sodium bicarbonate dipping. *Journal of Plant Pathology*, 91: 437–442.

Received: 2016–04–04

Accepted after corrections: 2017–02–23

Published online: 2017–04–07