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ORIGINAL ARTICLE

Biological Control of Postharvest Spoilage in Fresh Mandarins (*Citrus Reticulata* Blanco) Fruits Using Bacteria During Storage

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Abstract
Citrus fruit can be infected by many fungal pathogens, and these pathogens cause considerable losses during storage and transportation. Recently, biological control methods of postharvest diseases are getting more prevalent due to the effects of chemical residues on fruits. The present study was carried out to determine the antifungal activities of a total of four candidate biocontrol bacterial strains (*Bacillus subtilis* TV-6F, *B. subtilis* TV-17C, *Pseudomonas flourescens* RK-1105 and *Agrobacterium rubi* RK-33) against some pathogenic fungi (*Alternaria alternata, Sclerotinia sclerotiorum, Pythium ultimum, Penicillium digitatum* and *P. italicum*) on Petri plate assays. They were also tested to evaluate the potential application of the bacteria to bio-control postharvest decay on mandarin fruits during storage. In addition, they were tested to investigate the enzyme activities of the bacteria. The results of the present study showed that all non-pathogenic bacterial strains showed less or more antifungal activity against the tested pathogens on Petri plate assays. They were also significantly reduced disease severity on mandarin fruits during storage. Chitinase, glucanase and protease enzyme activities of the bacteria were positive except *A. rubi* RK-33. *B. subtilis* TV-17C and *A. rubi* RK-33 strains were the most effective bacteria. In controlling postharvest decay of mandarin fruit, these bacterial strains can be used as new bio-control agents. 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 strains on target organisms.

Keywords Bacillus · Bio-control · Citrus · Mandarin · Postharvest disease

Reduzierung von Nachernteverlusten bei Mandarinen durch den Einsatz von Bakterien (*Citrus reticulata* Blanco) während der Fruchtlagerung

Schlüsselwörter Bacillus · Biologischer Pflanzenschutz · Citrus · Mandarine · Nachernteverluste

Introduction

Citrus is grown commercially and produced in more than 137 countries around the world (Ismail and Zhang 2004). A global production of *Citrus* spp. (*Rutaceae*) exceeded 123 million tons in the world in 2013 (Palou et al. 2015). Mandarins (*Citrus reticulata* Blanco) are consumed in large quantities in Turkey and one of the most important exports of the citrus fruits.

Postharvest diseases have been identified as the greatest cause of post-harvest losses in fruits and vegetables and are caused significant economic losses (Prusky 2011). It is estimated that approximately 20–25% of the fruits and vegetables harvested due to action/attack by phytopathogenic microorganisms during post-harvest handling in developed countries (Gomes et al. 2015).

Citrus fruit can be infected by many fungal pathogens, and these pathogens cause considerable losses during storage and transportation (Maldonado et al. 2009). Losses are mainly caused by *Penicillium digitatum, P. italicum, Aspergillus flavus* and *Alternaria alternata* for citrus fruit (Eckert and Ogawa 1985; Diener et al. 1987; Whiteside et al. 1988; Palumbo et al. 2006). However, the importance

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and effect of these pathogens on the hesperidium industry are different from country to country. Therefore, first determine of importance of the fungal pathogens involved in postharvest decay and the spectrum for country are important.

Postharvest treatments such as thiabendazole (TBZ), imazalil (IMZ), sodium ortho-phenil phenate (SOPP) or other active ingredients have been used for many years. They still used in citrus packing houses to maintain fresh fruit, control postharvest decay, and extend fruit shelf life (Palou et al. 2015). Important problems such as environmental issues and health have been arisen for the citrus industry due to chemical residues or the occurrence of pathogenic resistant strains (Norman 1988; Wilson and Wisniewski 1989). Therefore the using of alternative postharvest control options such as natural plant extracts and biological agents have become important since it is realized as being environmentally safer (Janisiewicz and Korsten 2002).

Biological control is becoming an important alternative in the control against postharvest disease 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 (Rist and Rosenberg 1995; 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; Ketabchi et al. 2012; Elshafie et al. 2012; Mohammadi et al. 2014). They produce some extracellular lytic enzymes such as chitinase (Ordentlich et al. 1988), protease (Saligkarias et al. 2002) and glucanase (Wilson et al. 1991; Leelasuphakul et al. 2006). One of the most important mechanisms in phytopathogenic fungi biocontrol is degradation of fungal cell walls with bacterial hydrolytic enzymes (Weller 2007; Cherif et al. 1992).

This study aimed to determine the antifungal activities of the tested four candidate biocontrol bacterial strains (*B. subtilis* TV-6F, *B. subtilis* TV-17C, *Pseudomonas flourescens* RK-1105 and *Agrobacterium rubi* RK-33) against some pathogenic fungi (*Alternaria alternata, Sclerotinia sclerotiorum, Pythium ultimum, P. digitatum* and *P. italicum*) on Petri plate assays, to evaluate the potential application of the bacteria to control postharvest decay on mandarin fruits during storage, to investigate the enzyme activities of the bacteria, and to determine toxicological effect of them on fish.

Materials and Methods

Fruit Materials, Antagonistic Bacteria and Pathogenic Fungi

Fruit of mandarin (*Citrus reticulata* Blanco) cv. Clementine were used. Fruit were harvested from orchards of Netar Agriculture, Muğla, Turkey. Only healthy and commercially mature fruit were used in the experiments. Freshly harvested or briefly stored (no longer than 2 days) fruit 5 °C under dry conditions were used in the screening tests. Biocontrol bacterial strains and pathogenic fungus isolates were obtained from Ataturk University, Agricultural Faculty, Plant Protection Department culture collection. The pathogenic fungi were isolated from a decayed some citrus fruits and the biocontrol bacterial strains isolated from the phyllosphere and rhizosphere of wild and traditionally cultivated plants growing in Turkey (Eastern Anatolia Region). Bacterial cultures were maintained according to Tozlu et al. (2016).

Identification of the Bacteria and Fungi

The identity of all bacterial strains used in this study was confirmed according to fatty acid methyl esters (FAME) analysis by using Sherlock Microbial Identification System (Microbial ID, Newark, DE, USA) (Sasser 1990) and BIOLOG systems (Miller and Rhoden 1991; Holmes et al. 1994). The fungi were identified according to their ITS region. 200 mg were taken from the mycelia of the 15 pathogen-fungus isolates and 2 bio-agent fungus isolates, and added to 1 ml extraction buffer solution, which contained liquid nitrogen (0.2 M Tris 8.5, 0.25 M NaCl, 25 mM EDTA, 0.5% SDS). Then, phenol/chloroform purifier and ethanol precipitator were added, and diluted in DNA 50 µl TE buffer solution (10 mM Tris 7.5–1 mM EDTA). The DNA samples were clarified by using the ITS1 primer (TCCGTAGGTGAACCTGCGG) on ITS region 18S rDNA and the ITS4 primer (TCCTCCGCTTATTGATATGC) on 28S rRNA (White et al. 1990). The PCR was clarified at 50 µl reaction mix, which included 0.2 mM dNTP mix, 1.5 mM MgCl, 0.3 pmol from each primer, 1.5 U Taq polymerase, 1X polymerase buffer solution (100 mM Tris-HCl (pH 8.8 at 25 °C), 500 mM KCl, 0.8% (v/v) Nonidet P40) (Fermentas, Germany). The analysis was made at 94 °C in 1 s and at 30 a 94 °C 45 s + 50C 45 s + 72 °C 45 s. For separation, bromide was added in agarose gel and walked at ABI 3100 Genetic Analyzer, and left at UV. The sequence analysis was made at RefGen Company (Ankara, Turkey).

Bacterial Strains Hypersensitivity Tests

The biocontrol bacterial strains were tested on tobacco plants (*Nicotina tabacum* L. var. Samsun) for hypersensitivity as described by Klement et al. (1964).

Pathogenicity Test of the Fungi

Pathogenicity tests were exercised on mandarin fruits. The mandarin fruits surfaces were washed with tap water, sterilized with 70% ethanol and then washed sterilized distilled water (sdH_20). They were dried to being used for pathogenicity test. Fruits were dipped into a spore suspension of the fungus. The treatmented fruits were planted in polyethylene-lined plastic boxes, and stored in 80% humidity, at 10 °C under a photoperiod of 12-h light and 12-h dark. After incubation for 7 days, decay on the fruits surface and/or fungal mycelia growth or spore germination was determined as positive pathogenicity of tested fungus. sdH_2O alone was used as negative control. Each fungi was tested on 3 mandarin fruits.

Antifungal Assays on Petri Plate

The antifungal activity assays included cultures filtrates of the bacteria tested against pathogenic fungi on PDA medium. Fungi were grown in a PDA plate (90 mm) at 30 °C, in a 12 h light/dark cycle seven days before inoculation. Fresh culture of the each fungus were cut out with a cork borer (6 mm diameter), and placed in the center of 90 mm Petri plates with PDA medium. The bacteria were grown according to Tozlu et al. (2016). 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 control plate without any bacterial isolates. The fungal colonies diameter was recorded for antifungal activity, scored and measured as mm. All experiment treatments were performed twice with three replicates. The mycelial growth (mm) and growth inhibition percentage was calculated according to Mari et al. (1993) formula:

$$\textbf{Growth inhibition}\,(\%) = (\mathbf{C} - \mathbf{T}) \times \mathbf{100}/(\mathbf{C} - \mathbf{6})$$

C: pathogen colony diameter in the control group
T: pathogen diameter in the treatments colonies
6: pathogen disc diameter

In vivo Assays on Mandarin Fruits

The unscathed mandarin fruits were selected, previously untreated fungicide and as homogenous as possible in size and maturity, and were stored at 5 °C under dry conditions until use. The cell suspensions of the bacteria were adjusted to 1×10^8 cfu/ml in sdH_2O. Each mandarin fruit was dipped into the bacterial suspension. The fruits were placed in plastic boxes. They were stored in 80% humidity, at 10 °C, in a photoperiod of 12-h dark and 12-h light. The study was arranged in a randomized block design. Treatments were applied in three replicates. Fifty fruits were used in each experiments. Only sdH_2O was used as control. Decay on fruits' surface was measured 45 days later. The decay of each individual mandarin was evaluated on a 1–5 scale using a modified visual rating scale from Nunes et al. (2007).

Detection of Hydrolytic Enzymes of the Antagonistic Bacteria

Chitinase enzyme activities were determined according to Senol et al. (2014). The reaction mixture consisted of 0.2 mL suitably diluted enzyme solution and 0.5 mL 0.5% (w/v) of colloidal chitin (was prepared in 50 mM citrate buffer pH 6) was incubated at 37 °C for 30 min. The reaction was arrested by addition of 0.75 mL 3, 5 dinitrosalicylic acid (DNS) reagent (Miller 1959), followed by heating for 10 min in 80 °C water bath. The activity was determined by monitoring absorbance change at 540 nm. One unit of the enzyme activity was defined as the amount of enzyme that catalysed the release of 1 µmol mL^{-1} min^{-1} of reducing sugar under the above mentioned assay conditions. Glucanase activity was detected (using Lichenan (0.2%)) according to Teather and Wood method (Teather and Wood 1998). Determination of protease enzyme carried out on plates of Skim Milk Agar (SMA) medium and plates inoculated with bacteria were then incubated at 27 °C for 24 h (Atlas and Parks 1997). The colorless halo zone diameters (around the bacterial colonies) were measured to determine the ability of protease and glucanase production.

Statistical Analyses

All data was done by JUMP 5.0 in the present study and the means were separated by LS Means Tukey HSD tests.

Results

The list of bio-control bacterial strains and their hypersensitivity test results used in this study is presented in Table 1. Six bacterial strains consisted of four different genera including *Bacillus, Pseudomonas* and *Agrobacterium* accord-

Table 1 Identification results of biocontrol bacterial strains according to MIS and BIOLOG systems and their hypersensitivity test results on tobacco

Strains	MIS results	SIM	BIOLOG results	SIM	Isolated from	HR	References
RK-33	*Agrobacterium rubi*	0.786	*Agrobacterium rubi*	0.56	Apple	–	Kotan et al., 2005
RK-1105	*Pseudomonas flourescens*	0.673	*Pseudomonas flourescens*	0.77	Soil	–	In this study
TV-6F	*Bacillus subtilis*	0.831	*Bacillus subtilis*	0.56	Graminea	–	Cakmakcı et al. 2010
TV-17C	*Bacillus subtilis*	0.677	*Bacillus subtilis*	0.76	Raspberry	–	Cakmakcı et al. 2010

SIM Similarity index, *HR* Hypersensitivity test, – Negative reaction

Table 2 Identification results of pathogenic fungal isolates used in this study and their pathogenicity test results on mandarina

Isolate	ITS results	Identify (%)	Isolated from	PAT	ITC sequence[a]
DP-1	*Alternaria alternata*	0.99	Tomato	+	AF218791
DP-2	*Penicillium digitatum*	0.98	Orange	+	KX398668
DP-3	*Penicillium italicum*	0.98	Orange	+	DQ991463
DP-4	*Fusarium equiseti*	0.99	Tomato	+	KR094457
DP-5	*Sclerotinia sclerotiorum*	0.98	Cucumber	+	CP017820

[a]GenBank database *SIM* Similarity index, – Positive reaction

Table 3 The effect of bacterial applications on mycelial growth (mm) and growth inhibition (%) of pathogenic fungi treated with bacteria on Petri plate assays

Pathogenic fungus	Bacterial strains							
	TV-17C		RK-1105		RK-33		TV-6F	
	MG (mm)	GI (%)	MG (mm)	GI (%)	MG (mm)	GI (%)	MG (mm)	GI (%)
S. sclerotiorum	50	47	47	51	56	40	27	75
P. ultimum	28	73	50	47	50	47	45	53
A. alternata	32	69	20	83	50	47	23	79
P. digitatum	33	67	37	63	35	65	32	69
P. italicum	37	63	48	50	6	100	60	35

MG Mycelial growth, *GI* Growth inhibition

ing to both MIS and BIOLOG. Identification results of the bacteria (*A. rubi* RK-33, *B. subtilis* TV-17C and TV-6F and *P. flourescens* RK-1105) were the same according to MIS and BIOLOG. Similarity index of MIS and BIOLOG identification results were changed from 624 to 0.831 for MIS and from 0.56 to 0.76 for BIOLOG. Hypersensitivity test results of the all bacteria were negative.

The identification and pathogenicity test results of the pathogen fungus isolates were given in Table 2. DP-1 isolates were identified as *A. alternata*, DP-2 as *P. digitatum*, DP-3 as *P. italicum*, DP-4 as *P. ultimum* and DP-5 as *Sclerotinia* with respect to the identification test results. All the fungal isolates were pathogenic on mandarin fruits.

According to the results of antifungal assays conducted on Petri plates, all bacterial strains performed less or more antifungal activity against the tested pathogens (Table 3). The mycelial growth of the pathogenic fungi changed from 6 to 65 mm. Percentage of growth inhibition of the fungus treated with the bacterial strains changed from 35 to 100%. The most effective bacteria on mycelial growth of the pathogenic fungi were *B. subtilis* TV-6F strain for *S. sclerotiorum* (75%), *B. subtilis* TV-17C strain for *F. culmorum* (73%), *P. flourescens* RK-1105 strain for *A. alternata* (83%), *B. subtilis* TV-6F strain for *P. digitatum* (69%) and *A. rubi* RK-33 strain for *P. italicum* (100%) according to the control applications (Fig. 1).

The results of the enzyme activities are presented in Table 4. Chitinase, glucanase and protease enzyme activities of all bacteria were positive. The glucanase and protease enzyme activity of RK-1105 strain was not tested. The enzyme activities were changed from 65 to 97 for chitinase, from 26 to 32 for amylase and from 00 to 41 EU/mL. min for protease.

The results of in vivo assays are presented in Table 5. All of the tested bacteria significantly reduced decay on mandarin fruits' surface in comparison to the control not treated with bio-control bacteria. *B. subtilis* TV-6F was the most effective application (Fig. 2).

Discussion

During storage, fruits and vegetables are often subject to varying levels of microbial decay, mainly due to pathogenic fungi that usually infect the host through wounds sustained during harvest, handling and/or processing (El Ghaouth et al. 2002). The highest postharvest losses have been recorded on the export markets related to range of pathogens. These consist *Alternaria* rot caused by *Al-*

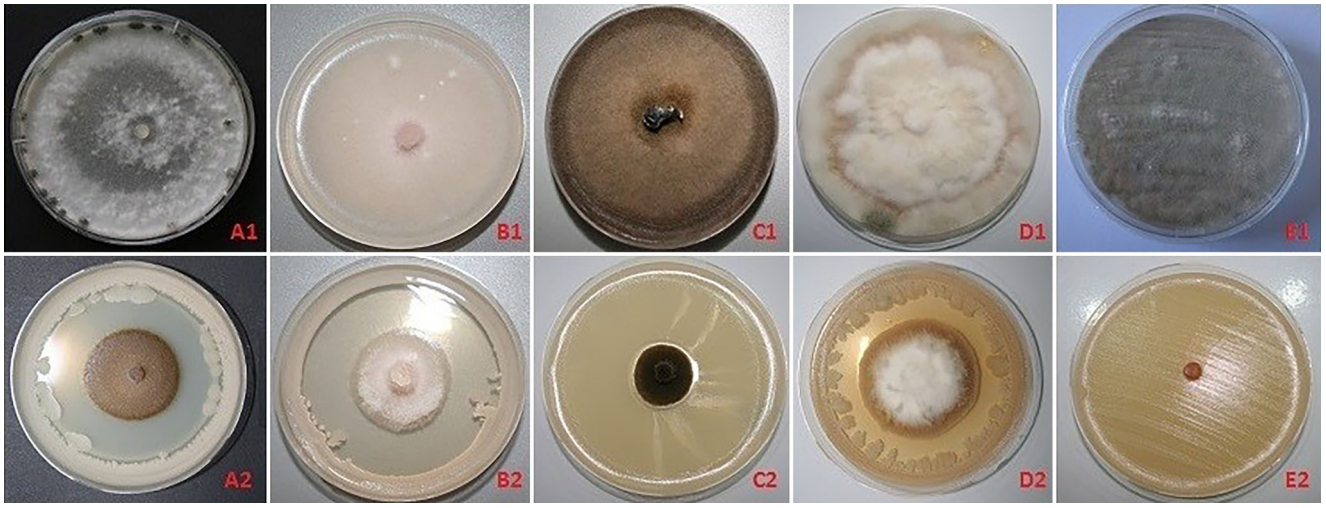

Fig. 1 The most effective bacterial strains on mycelial growth (mm) of pathogenic fungi on Petri plate assays. Only pathogen (*A1*: *S. sclerotiorum*, *B1*: *P. ultimum*, *C1*: *A. alternate*, *D1*: *P. digitatum*, *E1*: *P. italicum*) and pathogens treated bacteria (*A2*: *B. subtilis* TV-6F, B2: *B. subtilis* TV-17C, C2: *P. flourescens* RK-1105, D2: *B. subtilis* TV-6F, E2: *A. rubi* RK-33)

Fig. 2 The effect of the bacterial strains on decay on mandarin fruits' surface. *1*: *B. subtilis* TV-17C, *2*: *P. flourescens* RK-1105, *3*: *A. rubi* RK-33, *4*: *B. subtilis* TV-6F and *C*: Control (no bacteria)

ternaria citri (Whiteside et al. 1988), *Trichoderma* rot caused by *Trichoderma viride* (Whiteside et al. 1988), anthracnose caused by *Colletotrichum gloeosporioides* (Davies and Albrigo 1994), gray mould caused by *Botrytis cinerea* (Agrios 1997), green and blue mould caused by *Penicillium* species, *Aspergillus* rot caused by *Aspergillus niger*, brown rot caused by *Phytophthora parasitica, Diplodia* stem-end rot caused by *Diplodia natalensis* (Brown and Eckert 2000) and sour rot caused by *Geotrichum candidum* (Howard 1936).

Postharvest diseases are generally controlled by fungicides. The fruit surface is completely covered by the fungicides, and the residue is persistent for the life of the fruit (Holmes and Eckert 1999). However, these chemical agents have been applied for many years with few or limited success due to the development of resistance, their effect

Table 4 Enzyme activities of the bacterial strains used in this study

Bacterial strains	Enzyme activities of the bacteria (EU/mL.min)			References
	Chitinase	Glucanase	Protease	
B. subtilis TV-6F	65.0	26	30	Mohammadi et al. 2017
B. subtilis TV-17C	67.0	30	41	Mohammadi et al. 2017
A. radiobacter RK-33	85.0	32	08	In this study
P. flourescens RK-1105	97.0	Nt	Nt	In this study

Nt Not tested

Table 5 The effect of the applications on decay on mandarin fruits' surface

No	Applications	Decay index (1–5 scales)
1	*B. subtilis* TV-17C	1.41 B
2	*P. flourescens* RK-1105	1.13 BC
3	*A. rubi* RK-33	1.10 BC
4	*B. subtilis* TV-6F	1.07 C
5	Control	4.43 A
CV		48.00
LS means Tukey HSD		0.33

on human health, and generates environmental concerns mainly due to the carcinogenic and/or teratogenic properties of the compounds, and by their cumulative toxic effects (Thonglem et al. 2007; Pimenta et al. 2008). In this context, it is occurred that microbial biological control agents were shown important potential as an alternative to synthetic fungicides for diseases control of postharvest, and suggested an eco-friendly alternative to the synthetic pesticides (Kotan et al. 2009). A commercial bio-control product containing the yeast *Candida oleophila* has been available under the name Aspire (Ecogen Corporation, Langhorne, PA) in Israel and the United States for use against postharvest decay of citrus (Fravel 2005).

The present work was carried out to evaluate the antifungal activities of the bacterial strains including *B. subtilis* TV-17C, *B. subtilis* TV-6F, *Pseudomonas flourescens* RK-1105, and *Agrobacterium rubi* RK-33. All bacterial strains significantly decreased disease severity on fruits of mandarin during storage. The culture filtrates of *A. rubi* RK-33 and *B. subtilis* TV-6F strain were given the most successful results. In a previous study, we tested for antifungal activity of *B. subtilis* TV-6F and *B. subtilis* TV-17C strains against *S. sclerotiorum* on Petri plate assays and in pot assays. The results indicated that these bacterial strains *S. sclerotiorum* biocontrol agents in red cabbage (Tozlu et al. 2016). *B. subtilis* TV-17C strain may have positive effect on plant growth under unsuitable plant condition because of their high level of organic acid and amino acid contents (Gunes et al. 2015).

There are many studies showing that *Bacillus* occurring naturally on the surface of fruits or vegetables is used as a bio-control agent of postharvest diseases (Kotan et al. 2009; Ketabchi et al. 2012; Elshafie et al. 2012; Mohammadi et al. 2014). However, there is not any study showing that *A. rubi* is used as a bio-control agent of postharvest diseases. The first bacterium called *A. rubi* strain K 84 (currently strain K-1026) and produced Agrocin 84 antibiotic was 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. rubi* RK-33 strains being used for biological control of post-harvest plant diseases.

The bacteria included *Bacillus* genus have important potential as bio-control agents because they maintain their viability when stored for long time (Nagorska et al. 2007; Ongena and Jacques 2008). *Bacillus* species, particularly, *B. cereus, B. subtilis* and *B. amyloliquefaciens*, have been used in disease and pest management programs, successfully (Wulff et al. 2002; Obagwu and Korsten 2003; Nagorska et al. 2007; Ji et al. 2008; Francis et al. 2010). Considering the cases mentioned, gram-positive bacteria used in the natural formulation offer great advantage. Moreover, *B. subtilis* cells can produce dormant spores resistant to unsuitable conditions and thus can be easily formulated and stored (Piggot and Hilbert 2004).

Bacterial strains were important for antifungal activity because they produce some extracellular lytic enzymes such as protease (Saligkarias et al. 2002), glucanase (Leelasuphakul et al. 2006) and chitinase (Ordentlich et al. 1988). One of the most important mechanisms is the degradation of fungal cell walls with hydrolytic enzymes of bacterial strains (Elshafie et al. 2012; Weller 2007; Cherif et al. 1992). In addition, antibiotics and different biologically active compounds are produced by *B. subtilis.* These are able to induce systemic resistance (Stein 2005; Nagorska et al. 2007; Ongena and Jacques 2008). All strains used in this study were exhibited antifungal activity by producing diffusible metabolites correlated with their ability in produce extracellular hydrolytic enzymes. All tested bacterial strains produced chitinase, glucanase and protease enzyme (except *A. rubi* RK-33) reduced the growth rate of the pathogen and inhibited fungal growth. Results from the volatile metabolic assay suggested that cell wall degrading enzymes and metabolic substances may contribute to the inhibition of fungal growth. These substances may cause ultrastructure defects in fungal spores and hypha.

The environmental pollution and health hazards based on chemical use are increase continuously. These situations required the development of alternative strategies for postharvest citrus diseases control. The microbial antagonists and natural plant products defined as safety. These are suitable

to replace the fungucides (Smilanick et al. 2005; Sharma et al. 2009; Talibi et al. 2014).

In conclusion, this study showed that *B. subtilis* TV 6F and *A. rubi* RK-33 should use as potential bio-control agents to *P. digitatum*. These strains can be used as new bio-control agents for postharvest decay control 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 strains especially *B. subtilis* TV-6F on target organisms.

Conflict of interest E. Tozlu, M.Ş. Kotan, N. Tekiner, N. Dikbaş and R. Kotan declare that they have no competing interests.

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